




# *User Manual*

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## QuantiGene® ViewRNA ISH Tissue Assay

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# Introduction

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## About This Manual

This manual provides complete instructions for performing the QuantiGene ViewRNA ISH Tissue Assay for visualization of target mRNA in formalin-fixed paraffin-embedded (FFPE) samples prepared in accordance with the guidelines provided in this user manual.

This manual provides the following:

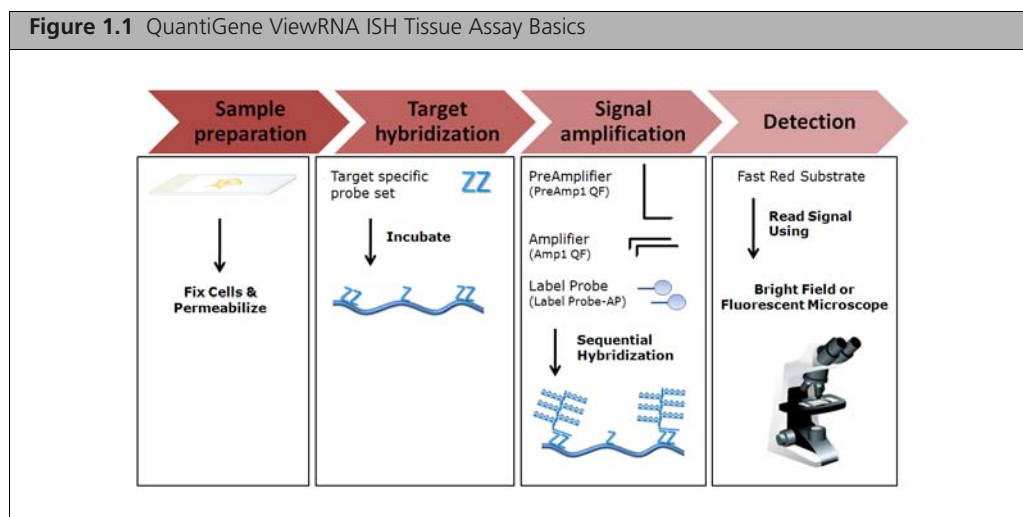
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## Assay Overview

*In situ* hybridization techniques are used to visualize DNA or RNAs within cells. However, the *in situ* analysis of RNA, in particular, has always been limited by low sensitivity and difficult probe synthesis. The QuantiGene® ViewRNA ISH Tissue Assay, based on branched DNA signal amplification technology, has the sensitivity and robustness to measure single-copy mRNA in single cells.

The assay is intended for chromogenic visualization of target mRNAs using a bright field microscope. The assay is compatible with fluorescent visualization; however the assay procedures were not optimized for this readout format. The assay protocol and reagents supplied are configured for processing a minimum of 12 slides if the 96 assay kit size was purchased, or processing a minimum of 6 slides at a time if the 24 assay kit size was purchased.

## How it Works



**Sample Preparation.** FFPE tissue sections are fixed, then treated to a Pretreatment Solution followed by Protease digestion to allow target accessibility.

**Target Hybridization.** A target-specific Probe Set hybridizes to the target RNA. Subsequent signal amplification is predicated on specific hybridization of adjacent Probe Set oligonucleotides indicated by “ZZ” in the image above. A typical Probe Set will contain 20 oligonucleotide pairs (“ZZ”), for simplicity only two are shown.

**Signal Amplification.** Signal Amplification is achieved via a series of sequential hybridization steps. The PreAmplifier molecule hybridizes to each pair of bound Probe Set oligonucleotides, then multiple Amplifier molecules hybridize to each PreAmplifier. Next, multiple Label Probe oligonucleotides conjugated to alkaline phosphatase hybridize to each Amplifier molecule. A fully assembled signal amplification “Tree” has 400 binding sites for the Label Probe.

**Detection.** Following the addition of the Fast Red Substrate, alkaline phosphatase breaks down the substrate to form a precipitate (red dots) that indicate the presence of the target RNA molecule. Target mRNA is visualized using a standard bright field or fluorescent microscope.

## Contacting Technical Support

For technical support, contact the appropriate resource provided below based on your geographical location. For an updated list of FAQs and product support literature, visit our website at [www.affymetrix.com/panomics](http://www.affymetrix.com/panomics).

**Table 1.1** Technical Support Contacts

Location	Contact Information
North America	1.877.726.6642 option 1, then option 3; pqbhelp@affymetrix.com
Europe	+44 1628-552550; techsupport_europe@affymetrix.com
Asia	+81 3 6430 430; techsupport_asia@affymetrix.com

## Required Materials

The QuantiGene ViewRNA ISH Tissue Assay is composed of the following 3 modules, each sold separately and available in multiple sizes:

- QuantiGene ViewRNA ISH Tissue Assay Kit
- QuantiGene ViewRNA Chromogenic Signal Amplification Kit
- QuantiGene ViewRNA TYPE 1 Probe Set(s)

### Recommended (but not required)

Two additional QuantiGene ViewRNA TYPE 1 Probe Sets, one to be used as a positive control and the second to be used to optimize the assay conditions. Refer to [Experimental Design Guidelines on page 7](#) for more information.

## QuantiGene ViewRNA ISH Tissue Assay Kit

QuantiGene ViewRNA ISH Tissue Assay Kits are available in two sizes: QVT0050 and QVT0051, sufficient for 24 or 96 assays, respectively. Each kit is configured for processing a minimum of 6 or 12 slides, respectively, per experiment.

The components of the QuantiGene ViewRNA ISH Tissue Assay Kit and their recommended storage conditions are listed below. Refer to the Package Insert for quantities of individual components supplied. Kits are shipped in 2 parts, based on storage conditions and have a shelf life of 6 months from date of delivery when stored as recommended.

**Table 1.2** Assay Kit Components and Their Storage Conditions

Component	Description	Storage
100X Pretreatment Solution	Aqueous buffered solution	2-8 °C
Protease QF <sup>a</sup>	Enzyme in aqueous buffered solution	2-8 °C
Probe Set Diluent QF	Aqueous solution containing formamide and detergent	2-8 °C
Amplifier Diluent QF	Aqueous solution containing formamide and detergent	2-8 °C
Label Probe Diluent QF	Aqueous solution containing detergent	2-8 °C
Wash Buffer Component 1 (Wash Comp 1)	Aqueous solution containing detergent	15-30 °C
Wash Buffer Component 2 (Wash Comp 2)	Aqueous buffered solution	15-30 °C

<sup>a</sup> **IMPORTANT!** Do not freeze.

## QuantiGene ViewRNA Chromogenic Signal Amplification Kits

QuantiGene ViewRNA Chromogenic Signal Amplification Kits are available in two sizes: QVT0200 and QVT0201, sufficient for 24 or 96 assays, respectively. Each kit is configured for processing a minimum of 6 or 12 slides, respectively, per experiment.

The components of the QuantiGene ViewRNA Chromogenic Signal Amplification Kit and their recommended storage conditions are listed below. Refer to the Package Insert for quantities of individual components supplied. Kits are shipped in 2 parts, based on storage conditions and have a shelf life of 6 months from date of delivery when stored as recommended.

**Table 1.3** Chromogenic Signal Amplification Kit Components and Storage Conditions

Component	Description	Storage
PreAmp1 QF	DNA in aqueous buffered solution	–20 °C
Amp1 QF	DNA in aqueous buffered solution	–20 °C
AP Enhancer Solution	Aqueous buffered solution	2–8 °C
Fast Red Tablets	Red precipitating substrate for the detection of alkaline phosphatase activity	2–8 °C
Naphthol Buffer	Buffer required for preparation of Fast Red Substrate	2–8 °C
Label Probe-AP	Alkaline Phosphatase-conjugated oligonucleotide in aqueous buffered solution	2–8 °C

## QuantiGene ViewRNA TYPE 1 Probe Sets

In addition to the QuantiGene ViewRNA ISH Tissue Assay Kit and the Chromogenic Signal Amplification Kit, QuantiGene ViewRNA TYPE 1 Probe Sets specific to your targets of interest must be purchased separately. Probe Sets are available in multiple sizes and should be stored at –20 °C. Refer to the Package Insert for more information.

**!** **IMPORTANT:** QuantiGene ViewRNA ISH Tissue Assay Kits are only compatible with QuantiGene ViewRNA TYPE 1 Probe Sets. Do not use other QuantiGene ViewRNA TYPE Probe Sets.

**Table 1.4** ViewRNA Probe Set and Storage Conditions

Component	Description	Storage
QuantiGene ViewRNA TYPE 1 Probe Set	RNA-specific oligonucleotides to your target of interest and compatible with the TYPE 1 Signal Amplification system comprised of: PreAmp1 QF, Amp1 QF, and Label Probe-AP	–20 °C

## Required Materials Not Provided

Other materials required to perform the QuantiGene ViewRNA ISH Tissue Assay that are not included in the QuantiGene ViewRNA ISH Tissue Assay Kit or the Chromogenic Signal Amplification Kit are listed here.

**!** **IMPORTANT:** When specified, do not use alternate materials or suppliers.



Table 1.5 Required Materials Not Provided

Required Material	Source	Part Number or Model
Double-distilled water (ddH <sub>2</sub> O)	Major laboratory supplier (MLS)	
95% Ethanol	VWR	89015-512
10X PBS, pH 7.2-7.4	Bio-Rad Laboratories or Invitrogen	161-0780 70013-032
Gill's Hematoxylin I	American Master Tech Scientific	HXGHE1LT
HistoClear	National Diagnostics	HS-200
37% Formaldehyde	Fisher Scientific	F79-1
<b>WARNING: Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.</b>		
27-30% Ammonium Hydroxide	VWR	JT9726-5
<b>WARNING: Ammonium hydroxide is Highly volatile. Use in fume hood.</b>		
ImmEdge Hydrophobic Barrier Pen	Vector Laboratories	H4000
UltraMount	DAKO	S1964
Tissue Tek Staining Dish (clear color), 3 required	American Master Tech Scientific	LWT4457EA
Tissue Tek Clearing Agent Dish (green color), 1 required	American Master Tech Scientific	LWT4456EA
Tissue Tek Vertical 24 Slide Rack, 1 required	American Master Tech Scientific	LWSRA24
ThermoBrite Hybridization Chamber capable of maintaining 40 ± 1 °C	Abbot Molecular	07J91-010 (110V)
QuantiGene View Temperature Validation Kit	Affymetrix	QV0523
Humidifying Strips	Abbot Molecular	30-144115
Water-proof remote probe thermometers, validated for 95-100 °C	VWR	46610-024
1000 mL Glass Beaker	MLS	
Cover Glass, 24 mm x 55 mm	VWR	48382-138
Forceps	MLS	
Pipettes, P20, P200, P1000	MLS	
Fume hood	MLS	
Isotemp Hot Plates	Fisher Scientific	11-300-49SHP (120V) 11-302-49SHP (230V)
Table-top microtube centrifuge	MLS	
Water Bath capable of maintaining 40 ± 1 °C	MLS	
Microscope and imaging instruments	See <a href="#">Appendix E</a>	

**Table 1.5** Required Materials Not Provided

Required Material	Source	Part Number or Model
<i>Optional.</i> Dry incubator or oven utilizing horizontal airflow and capable of maintaining 80 °C <sup>a</sup>	Affymetrix	QS0700, QS0701 (120V) or QS0710, QS0711 (220V)
<i>Optional.</i> Temperature/humidity meter (if using tissue culture incubator) <sup>a</sup>	Fisher-Scientific	11-661-19
<i>Optional.</i> Tissue culture incubator, 40 ± 1 °C without CO <sub>2</sub> and 65% humidity <sup>a</sup>	Napco VWR	51201082 9150860
<i>Optional.</i> Aluminum slide rack for use in tissue culture incubator, quantity 3 <sup>a</sup>	VWR	100493380
<i>Optional.</i> Fluorescent microscope with 10X eyepiece and 20X or 40X objective and Ex556, Em573 filter set (Cy3) <sup>b</sup>	MLS	
<i>Optional.</i> DAPI <sup>b</sup>	Invitrogen	D3571

<sup>a</sup> Alternative equipment required if ThermoBrite Incubator is not available.

<sup>b</sup> Additional equipment and reagent necessary for fluorescence detection.

## Assay Guidelines

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### Tissue Preparation Guidelines

The following are guidelines for FFPE sample preparation. Samples prepared outside of these guidelines may not produce the best results.

- Fix freshly-dissected tissues with 10% neutral-buffered formalin for 16-24 hr at room temperature
- Rinse, dehydrate, and embed (in paraffin) fixed samples
- Cut FFPE sections to a thickness of 4-6  $\mu\text{m}$
- Mount a single section onto a positively charged glass slide (Superfrost Plus, Fisher Scientific P/N 12-550-15)
- Analyze sample sections within 3 months of sectioning when stored at room temperature (20-25 °C) under desiccation



**NOTE:** The QuantiGene ViewRNA ISH Tissue Assay is not intended for use with slides containing multiple sections or tissue microarrays.

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### Experimental Design Guidelines

#### Assay Controls

We recommend running one positive and one negative control slide, based on your sample type, in every QuantiGene ViewRNA ISH Tissue Assay. This will allow you to qualify your results as well as to minimize false negatives.

#### Negative Control

This slide undergoes the entire assay procedure with the Probe Set omitted. This control will assess the assay background.

#### Positive Control

This slide is processed using a Probe Set for a target that has consistent, high to medium-high homogenous expression in your sample type. This control ensures the assay procedure has been run successfully.

#### Replicates

We recommend running all assays in duplicate.

#### Recommended Pretreatment Assay Optimization

When working with a new tissue type, we recommend performing the pretreatment assay optimization procedure as described in [Pretreatment Assay Optimization Procedures and Typical Results on page 31](#) to identify the optimal pretreatment boiling time and protease digestion time to un-mask the mRNA. Applying the optimal condition will not only provide a favorable environment for the QuantiGene ViewRNA Probe Set to bind to the target mRNA but will also have an impact on the final chromogenic staining quality and tissue morphology. The same optimal condition can be used for different Probe Sets.

If you choose to bypass the recommended pretreatment assay optimization, you can use the conditions for your specific tissue type as described in [Pretreatment Assay Optimization Lookup Table on page 43](#) for a general guideline. If you are using multiple tissue types with different optimal conditions, we recommend you try each condition and choose the one that works best for your samples. If you do not obtain the desired results, we recommend performing the pretreatment assay optimization procedure.



## Assay Workflow

### About the Assay Workflow

The QuantiGene ViewRNA ISH Tissue Assay can be run in a single long day or broken up over 2 days for added flexibility. There is an optional assay stop point, following the target probe hybridization, where the assay can be stopped and work can be continued the following day.

For convenience, the procedure is sectioned into two parts:

- Part 1: Sample Preparation and Target Probe Set Hybridization
- Part 2: Signal Amplification and Detection

with the stop point at the end of Part 1. We do not recommend stopping at any other point in the assay.

Below is an overview of the assay procedure.

### Part 1: Sample Preparation and Target Probe Hybridization

Table 3.1 Part 1 Assay Workflow

Step	Task
<b>Step 1. Bake Slides</b>  35 min	Bake slides for 30 min at $60 \pm 1$ °C
<b>Step 2. Prepare Buffers and Reagents while Slides Bake</b>	<ul style="list-style-type: none"> <li>■ Prepare 2 L 1X PBS, 200 mL 10% formaldehyde in 1X PBS, 200 mL 4% formaldehyde in 1X PBS, 3 L Wash Buffer, 500 mL 1X Pretreatment Solution, (optional) 500 mL Storage Buffer</li> <li>■ Ensure availability of 400 mL 95% ethanol, 400 mL ddH<sub>2</sub>O, 200 mL HistoClear</li> <li>■ Prewarm 40 mL 1X PBS</li> <li>■ Thaw Probe Set(s) on ice</li> </ul>
<b>Step 3. Fix Slides</b>  1 hr 5 min	<ul style="list-style-type: none"> <li>■ Fix slides in 10% formaldehyde for 1 hr at RT (under a fume hood)</li> <li>■ Rinse slides in 1X PBS</li> <li>■ Decant 1X PBS from slides and let air dry</li> </ul>
<b>Step 4. Deparaffinization</b>  30 min	<ul style="list-style-type: none"> <li>■ Bake slides for 3 min at <math>80 \pm 1</math> °C</li> <li>■ Incubate in HistoClear for 10 min at RT (under a fume hood)</li> <li>■ Rinse 2X with 95% ethanol</li> <li>■ Decant 95% ethanol from slide and let air dry</li> </ul>
<b>Step 5. Draw Hydrophobic Barrier</b>  1 hr	<ul style="list-style-type: none"> <li>■ Create hydrophobic barrier</li> <li>■ Allow slides to air dry for 20-30 min at RT</li> </ul>
<b>Step 6. Tissue Pretreatment</b>  10-25 min, depending on optimized time	<ul style="list-style-type: none"> <li>■ Boil slides in 1X Pretreatment Solution at 95-100 °C for the optimal time determined in the pretreatment assay optimization procedure</li> <li>■ Rinse slides in ddH<sub>2</sub>O</li> </ul>

Table 3.1 Part 1 Assay Workflow

Step	Task
<b>Step 7. Protease Digestion and Fixation</b>  30-50 min depending on optimized time	<ul style="list-style-type: none"> <li>■ Prepare Working Protease Solution</li> <li>■ Add prewarmed 1X PBS to slides, incubate for 3 min at <math>40 \pm 1^\circ\text{C}</math></li> <li>■ Decant 1X PBS and add Working Protease Solution, incubate at <math>40 \pm 1^\circ\text{C}</math> for optimal time determined in the pretreatment assay optimization procedure</li> <li>■ Rinse slides in 1X PBS</li> <li>■ Incubate in 4% formaldehyde for 5 min at RT</li> <li>■ Rinse slide in 1X PBS</li> <li>■ Save 4% formaldehyde solution for use in Part 2</li> </ul>
<b>Step 8. Target Probe Set Hybridization</b>  3 hr 10 min	<ul style="list-style-type: none"> <li>■ Prepare Working Probe Set Solutions</li> <li>■ Add Working Probe Set and incubate for 3 hr at <math>40 \pm 1^\circ\text{C}</math></li> </ul>
<b>Step 9. Wash Slides</b>  10 min	Wash slides in Wash Buffer, 3 times for 2 minutes each
<b>Step 10. Optional Stop Point</b>  1 min	<ul style="list-style-type: none"> <li>■ Store slides in Storage Buffer at RT. Do not exceed 24 hr.</li> <li>■ Store 4% formaldehyde, 1X PBS, and Wash Buffer at RT for use in Part 2</li> </ul>

## Part 2: Signal Amplification and Detection

Table 3.1 Part 2 Assay Workflow

Step	Task
<b>Step 11. Preparation for Part 2</b>  5 min	Remove slides in Storage Buffer, and wash with Wash Buffer
<b>Step 12. Prepare Additional Buffers and Reagents</b>  10 min	<ul style="list-style-type: none"> <li>■ Prepare 0.01% ammonium hydroxide in ddH<sub>2</sub>O</li> <li>■ Ensure availability of 7 mL Gills Hematoxylin</li> <li>■ <i>Optional.</i> prepare 200 mL of DAPI</li> <li>■ Prewarm Amplifier Diluent QF and Label Probe Diluent QF to <math>40 \pm 1^\circ\text{C}</math></li> <li>■ Thaw PreAmp1 QF and Amp1 QF, store on ice</li> <li>■ Place Label Probe-AP on ice</li> <li>■ Bring Fast Red Tablets, Naphthol Buffer and AP Enhancer to RT</li> </ul>
<b>Step 13. PreAmplifier Hybridization</b>  35 min	<ul style="list-style-type: none"> <li>■ Prepare Working PreAmp1 Solution</li> <li>■ Add Working PreAmp1 Solution to slides, incubate at <math>40 \pm 1^\circ\text{C}</math> for 25 min</li> </ul>
<b>Step 14. Wash Slides</b>  10 min	Wash slides in Wash Buffer, 3 times for 2 minutes each
<b>Step 15. Amplifier Hybridization</b>  20 min	<ul style="list-style-type: none"> <li>■ Prepare Working Amp1 Solution</li> <li>■ Add Working Amp1 Solution to slides, incubate at <math>40 \pm 1^\circ\text{C}</math> for 15 min</li> </ul>

Table 3.1 Part 2 Assay Workflow

Step	Task
<b>Step 16. Wash Slides</b>  10 min	Wash slides in Wash Buffer, 3 times for 2 minutes each
<b>Step 17. Label Probe-AP Hybridization</b>  25 min	<ul style="list-style-type: none"> <li>■ Prepare Working Label Probe-AP Solution</li> <li>■ Add Working Label Probe-AP Solution to slides, incubate at <math>40 \pm 1</math> °C for 15 min</li> </ul>
<b>Step 18. Wash Slides</b>  10 min	Wash slides in Wash Buffer, 3 times for 3 minutes each
<b>Step 19. Apply Fast Red Substrate</b>  1 hr	<ul style="list-style-type: none"> <li>■ Incubate AP-Enhancer for 5 min at RT</li> <li>■ Decant then incubate with Fast Red Substrate for 30 min at <math>40 \pm 1</math> °C</li> <li>■ Rinse slides in 1X PBS</li> <li>■ Fix in 4% formaldehyde for 5 min at RT (under a fume hood)</li> <li>■ Rinse slides in 1X PBS</li> </ul>
<b>Step 20. Counter Stain</b>  50 min	<ul style="list-style-type: none"> <li>■ Incubate with Gill's Hematoxylin stain for 5 min at RT</li> <li>■ Rinse slides in ddH<sub>2</sub>O</li> <li>■ 0.01% ammonium hydroxide for 10 sec at RT</li> <li>■ Rinse slides in ddH<sub>2</sub>O</li> <li>■ Optional: DAPI for 1 min at RT</li> <li>■ Rinse slide in ddH<sub>2</sub>O</li> <li>■ Let slides air dry for 20 min at RT</li> </ul>
<b>Step 21. Add Cover-slip</b>  20 min	<ul style="list-style-type: none"> <li>■ Mount with Dako Ultra Mount and cover glass</li> <li>■ Observe under bright field or fluorescent microscope</li> </ul>





## QuantiGene ViewRNA ISH Tissue Assay Procedure

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### About the QuantiGene ViewRNA ISH Tissue Assay Procedure

The QuantiGene ViewRNA ISH Tissue Assay can be run in a single long day or broken up over 2 days for added flexibility. There is an optional assay stop point, following the target probe hybridization, where the assay can be stopped and work can be continued the following day.

For convenience, the procedure is sectioned into two parts:

- Part 1: Sample Preparation and Target Probe Set Hybridization
- Part 2: Signal Amplification and Detection

with the stop point at the end of Part 1. We do not recommend stopping at any other point in the assay.

The assay is optimized for using a ThermoBrite. If you do not have access to a ThermoBrite, use recommended alternative equipment, dry oven and tissue culture incubator without CO<sub>2</sub>. For a complete Assay Procedure refer to [QuantiGene ViewRNA ISH Tissue Assay Procedure using a Dry Oven and Humidified Incubator](#) on page 45.


### Important Procedural Notes and Guidelines

- Perform all steps in a well-ventilated area at room temperature unless otherwise noted.
- Procedure assumes running a maximum of 12 slides at a time.
- Maximum tissue area for this procedure is 30 mm x 22 mm.
- The QuantiGene ViewRNA ISH Tissue Assay is optimized for chromogenic visualization under bright field microscope. Detection using a fluorescent microscope may require additional optimization.
- Do not mix and match kit components from different lots.
- Before beginning procedure, know the pretreatment boiling time and protease digestion time (optimized conditions) for your sample type. If you do not know these optimized conditions, refer to [Pretreatment Assay Optimization Procedures and Typical Results](#) on page 31.
- Use a pencil to label all slides.
- Always use freshly prepared solutions in the Tissue Tek Staining and Clearing Agent dishes. Do not save or reuse solutions unless directed to do so (for example with the 4% formaldehyde solution).
- The Tissue Tek Staining Dish (clear) will be referred to as the “clear staining dish” throughout the assay procedure.
- The Tissue Tek Clearing Agent Dish (green) will be referred to as the “green clearing agent dish” throughout the assay procedure.
- Throughout the procedure, dedicate one clear staining dish for fixing in formaldehyde (we recommend labeling this dish). The other two clear staining dishes can be used interchangeably for: 1X PBS, 95% Ethanol, Wash Buffer and Storage Buffer. Rinse staining dishes in between steps with ddH<sub>2</sub>O.
- Validate the temperature of the ThermoBrite Incubator using the QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
- Unless otherwise stated, all reagents prepared should be stored at room temperature until use.
- Typical processing times included in the assay procedure assume that preparation for the following step is being done during the incubation periods.
- Before opening reagents supplied in the microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- Discard all reagents in accordance with local, state, and federal laws.

## Part 1: Sample Preparation and Target Probe Hybridization

### Part 1 Procedure

Step	Action
<b>Step 1. Bake Slides</b>  35 min	<p><b>A.</b> Use a pencil to label the slides.</p> <p><b>B.</b> Set ThermoBrite Incubator at <math>60 \pm 1^\circ\text{C}</math> and bake the slides for 30 min with the lid open.</p> <hr/> <p><b>NOTE:</b> This increases tissue attachment to the slide.</p> <hr/>
<b>Step 2. Prepare Buffers and Reagents While Slides Bake</b>  0 min	<p><b>A.</b> Prepare 2 L of 1X PBS: To a 2 L container add 200 mL of 10X PBS and 1.8 L ddH<sub>2</sub>O.</p> <p><b>B.</b> Prepare 10% formaldehyde in 1X PBS under a fume hood:</p> <hr/> <p><b>WARNING:</b> Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.</p> <hr/> <p>To a 200 mL capacity container add 146 ml 1X PBS and 54 mL of 37% formaldehyde and mix well.</p> <p><b>C.</b> Prepare 4% formaldehyde in 1X PBS: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well.</p> <p><b>D.</b> Prepare 3 L of Wash Buffer: To a 3 L capacity container add components in the following order to prevent precipitation from forming and then mix well: 2.5 L ddH<sub>2</sub>O, 27 mL Wash Comp 1, 7.5 mL Wash Comp 2, and ddH<sub>2</sub>O to 3 L.</p> <p><b>E.</b> Ensure availability of:</p> <ul style="list-style-type: none"> <li>■ 400 mL 95% ethanol</li> <li>■ 400 mL ddH<sub>2</sub>O</li> <li>■ 200 mL HistoClear</li> </ul> <p><b>F.</b> Pour solutions into Tissue Tek dishes:</p> <ul style="list-style-type: none"> <li>■ In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish</li> <li>■ In a fume hood, pour 200 mL of HistoClear into green Clearing Agent Dish</li> <li>■ Pour 200 mL of 95% ethanol into clear staining dish</li> <li>■ Pour 200 mL of 1X PBS into clear staining dish</li> </ul> <p><b>G.</b> Prewarm 40 mL of 1X PBS and Probe Set Diluent QF to <math>40 \pm 1^\circ\text{C}</math>.</p> <p><b>H.</b> Thaw Probe Set(s). Place on ice until use.</p> <p><b>I.</b> Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH<sub>2</sub>O.</p> <p><b>J.</b> Prepare 500 mL of Storage Buffer (for Optional Stop Point): To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH<sub>2</sub>O and mix well.</p>
<b>Step 3. Fix Slides</b>  1 hr 5 min	<p><b>A.</b> Insert slides into an empty slide rack and submerge into a clear staining dish containing 10% formaldehyde. Incubate for 1 hour at room temperature (RT) under a fume hood.</p> <p><b>B.</b> Remove the slide rack from the 10% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min. with frequent agitation.</p> <p><b>C.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min. with frequent agitation.</p> <p><b>D.</b> Remove each slide and decant the 1X PBS by flicking and placing it on its edge on a laboratory wipe. Place the slides on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.</p> <p><b>E.</b> Set ThermoBrite Incubator to <math>80^\circ\text{C}</math>.</p>

Step	Action
<b>Step 4. Deparaffinization</b>  <b>30 min</b>	<p><b>A.</b> Insert an empty slide rack into a green clearing agent dish containing 200 mL HistoClear solution. Cover the dish with the lid and bring it next to the ThermoBrite Incubator.</p> <p><b>B.</b> Bake the slides on the ThermoBrite Incubator with the lid open at <math>80 \pm 1^\circ\text{C}</math> for 3 min. The paraffin should melt as soon as the slides are on the ThermoBrite Incubator.</p> <p><b>C.</b> Remove lid from green Tissue Tek dish, and immediately insert the warm slides in the HistoClear solution, cover the dish with the lid and incubate under a fume hood at RT for 10 min. with frequent agitation.</p> <p><b>D.</b> Lift the slide rack from the HistoClear solution and submerge into the clear staining dish containing 200 mL 95% ethanol and incubate for 1 min. with frequent agitation.</p> <p><b>E.</b> Decant the 95% ethanol, refill with 200 mL of fresh 95% ethanol, and incubate for 1 min. with frequent agitation.</p> <p><b>F.</b> Remove the slides from the slide rack and place them on a paper towel to air dry for 5 min at RT. Discard the 95% ethanol.</p>
<b>Step 5. Draw Hydrophobic Barrier</b>  <b>1 hr</b>	<p><b>A.</b> Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.</p> <p><b>B.</b> To create a hydrophobic barrier, place the slide over the template image below, tissue sections should fall inside blue rectangle, and lightly trace the blue rectangle 2 to 4 times with the ImmEdge Hydrophobic Barrier Pen. It may be necessary to draw the hydrophobic barrier over tissue edges for larger sections or sections mounted close to the edge of the slide. Allow for barrier to dry at RT for 20-30 min.</p> <hr/> <p><b>IMPORTANT: Consistently draw hydrophobic barrier size as indicated in template, even if using smaller tissue sections.</b></p> <hr/> <p><b>IMPORTANT: Draw the barrier 2-4 times to ensure a solid seal.</b></p> <hr/> 
<b>Step 6. Tissue Pretreatment</b>  <b>10-25 min, depending on optimized time</b>	<p><b>A.</b> Bring 500 mL of 1X Pretreatment Solution to boil (<math>100^\circ\text{C}</math>) in a 1 L beaker tightly covered with aluminum foil on a hot plate. When boiling is reached, use a water-proof probe thermometer to measure and maintain the boiling temperature at <math>95\text{-}100^\circ\text{C}</math>.</p> <p><b>B.</b> Load the slides into the slide rack.</p> <p><b>C.</b> Using a pair of forceps, submerge the slide rack into the boiling 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate at <math>95\text{-}100^\circ\text{C}</math> for the optimal time as determined in <a href="#">Pretreatment Assay Optimization Procedures and Typical Results on page 31</a>.</p> <p><b>D.</b> Using a pair of forceps, remove the slide rack loaded with slides and submerge it into a clear staining dish containing 200 mL ddH<sub>2</sub>O. Incubate for 1 min with frequent agitation.</p> <p><b>E.</b> Decant the ddH<sub>2</sub>O and refill the clear staining dish with fresh ddH<sub>2</sub>O. Incubate for 1 min. with frequent agitation.</p> <hr/> <p><b>IMPORTANT: From this point forward do not let the tissue sections dry out.</b></p> <hr/>

Step	Action										
<b>Step 7. Protease Digestion and Fixation</b>  <b>30-50 min, depending on optimized time</b>	<p><b>A.</b> Set the ThermoBrite Incubator to 40 °C and insert two wet humidifying strips.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working Protease Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Protease QF</td><td>4 µL</td></tr> <tr> <td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Remove slides from slide rack, flick off excess ddH<sub>2</sub>O and place them onto a paper towel.</p> <p><b>D.</b> Add 500 µL of prewarmed 1X PBS to each slide and incubate on the ThermoBrite Incubator with the lid open for 3 min. at 40 ± 1 °C.</p> <p><b>E.</b> Working with one slide at a time, decant the 1X PBS, place the slide onto the ThermoBrite Incubator, and then add 400 µL of the Working Protease Solution onto the tissue section. Repeat for the rest of the slides.</p> <hr/> <p><b>IMPORTANT: Make sure every slide is incubated for the full duration of time determined by the Pretreatment Assay Optimization Procedure. Process ONE slide at a time to avoid drying.</b></p> <hr/> <p><b>F.</b> Close the lid and incubate at 40 ± 1 °C for the optimal time as determined in the <a href="#">Pretreatment Assay Optimization Procedures and Typical Results on page 31</a>.</p> <p><b>G.</b> After incubation, decant the Working Protease Solution from the slides, insert them into the slide rack and transfer slide rack into clear staining dish with 200 mL 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>H.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>I.</b> Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.</p> <p><b>J.</b> Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.</p> <p><b>K.</b> Transfer the slide rack from the 4% formaldehyde solution to the clear staining dish containing 1X PBS, and incubate for 1 min. with frequent agitation.</p> <p><b>L.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>M.</b> Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.</p>	Working Protease Solution per Slide		Reagent	Volume	Protease QF	4 µL	1X PBS (prewarmed to 40 °C)	396 µL	Total volume	400 µL
Working Protease Solution per Slide											
Reagent	Volume										
Protease QF	4 µL										
1X PBS (prewarmed to 40 °C)	396 µL										
Total volume	400 µL										

Step	Action																				
<b>Step 8. Target Probe Set Hybridization</b>  <b>3 hr 10 min</b>	<p><b>A.</b> Set the ThermoBrite Incubator to 40 °C and rewet the humidifying strips with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Probe Set Solutions by diluting the QuantiGene ViewRNA Probe Set(s) 1:50 in prewarmed Probe Set Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <hr/> <p><b>IMPORTANT: We recommend running assay controls, 1 positive and 1 negative control slide, to ensure proper signal is obtained.</b></p> <hr/> <table><tr><th>Working Probe Set Solution per Slide</th><th>Target Sample</th><th>Negative Control</th><th>Positive Control</th></tr><tr><th>Reagent</th><th colspan="3">Volume</th></tr><tr><td>Probe Set Diluent (prewarmed to 40 °C)</td><td>392 µL</td><td>400 µL</td><td>392 µL</td></tr><tr><td>QuantiGene ViewRNA TYPE 1 Probe Set</td><td>8 µL<sup>a</sup></td><td>0<sup>b</sup></td><td>8 µL<sup>c</sup></td></tr><tr><td>Total volume</td><td>400 µL</td><td>400 µL</td><td>400 µL</td></tr></table> <p><sup>a</sup> Use target Probe Set</p> <p><sup>b</sup> Do not include a target Probe Set</p> <p><sup>c</sup> Use positive control Probe Set</p> <p><b>C.</b> Remove each slide from 1X PBS and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe.</p> <p><b>D.</b> Place the slides flat on the lab bench and immediately add 400 µL Working Probe Set Solution to each tissue section.</p> <p><b>E.</b> Place the slides in the ThermoBrite Incubator, close the lid and incubate at 40 ± 1 °C for 3 hr.</p>	Working Probe Set Solution per Slide	Target Sample	Negative Control	Positive Control	Reagent	Volume			Probe Set Diluent (prewarmed to 40 °C)	392 µL	400 µL	392 µL	QuantiGene ViewRNA TYPE 1 Probe Set	8 µL <sup>a</sup>	0 <sup>b</sup>	8 µL <sup>c</sup>	Total volume	400 µL	400 µL	400 µL
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Total volume	400 µL	400 µL	400 µL																		
<b>Step 9. Wash Slides</b>  <b>10 min</b>	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Probe Set Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>																				
<b>Step 10. Optional Stopping Point</b>  <b>1 min</b>	<p><b>A.</b> Store slides in a clear staining dish containing 200 mL of Storage Buffer for up to 24 hours at RT.</p> <p><b>B.</b> The following reagent preparations should be stored at RT for use in Part 2:</p> <ul style="list-style-type: none"><li>■ 4% formaldehyde</li><li>■ 1X PBS</li><li>■ Wash Buffer</li></ul> <p><b>C.</b> All other reagent and solution preparations should be discarded.</p> <p><b>D.</b> When you are ready to continue the assay, proceed to <a href="#">Step 12. Prepare Additional Buffers and Reagents on page 18.</a></p>																				

## Part 2: Signal Amplification and Detection

### Part 2 Procedure

Step	Action										
<b>Step 11. Preparation for Part 2</b>  5 min	<p>If you paused the assay after Part 1, complete this step, otherwise go to <a href="#">Step 12. Prepare Additional Buffers and Reagents on page 18.</a></p> <p><b>A.</b> Remove the slides from Storage Buffer, transfer slide rack to clear staining dish containing Wash Buffer, and incubate for 1 min with frequent agitation.</p> <p><b>B.</b> Decant Wash Buffer, refill with 200 mL fresh Wash Buffer, and incubate for 1 min. with frequent agitation.</p>										
<b>Step 12. Prepare Additional Buffers and Reagents</b>  10 min	<p><b>A.</b> Prepare 1 L of 0.01% ammonium hydroxide in ddH<sub>2</sub>O: In a fume hood, add 0.33 mL 30% ammonium hydroxide to 999.67 mL ddH<sub>2</sub>O and mix well.</p> <p><b>B.</b> Ensure availability of 7 mL Gill's Hematoxylin.</p> <p><b>C.</b> If you plan on using fluorescent detection, prepare 200 mL DAPI. The final dilution of DAPI should be 0.5 µg/mL in 1X PBS.</p> <p><b>D.</b> Prewarm Amplifier Diluent QF and Label Probe Diluent QF buffers to 40 °C.</p> <p><b>E.</b> Thaw PreAmp1 QF and Amp1 QF. Place on ice until use.</p> <p><b>F.</b> Place Label Probe-AP on ice.</p> <p><b>G.</b> Bring Fast Red Tablets, Naphthol Buffer and AP Enhancer Solution to RT.</p>										
<b>Step 13. PreAmplifier Hybridization</b>  35 min	<p><b>A.</b> Set the ThermoBrite Incubator to 40 °C and rewet the humidifying strips with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working PreAmp1 Solution by diluting PreAmp1 QF 1:100 in prewarmed Amplifier Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working PreAmp1 Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Amplifier Diluent QF (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>PreAmp1 QF</td><td>4 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Remove each slide and decant the Wash Buffer by flicking and placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench and immediately add 400 µL of Working PreAmp1 Solution to each tissue section.</p> <p><b>D.</b> Place slides in the ThermoBrite Incubator. Close the lid and incubate at 40 ± 1 °C for 25 min.</p> <p><b>IMPORTANT: Incubation time should not exceed 25 min.</b></p>	Working PreAmp1 Solution per Slide		Reagent	Volume	Amplifier Diluent QF (prewarmed to 40 °C)	396 µL	PreAmp1 QF	4 µL	Total volume	400 µL
Working PreAmp1 Solution per Slide											
Reagent	Volume										
Amplifier Diluent QF (prewarmed to 40 °C)	396 µL										
PreAmp1 QF	4 µL										
Total volume	400 µL										
<b>Step 14. Wash Slides</b>  10 min	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working PreAmp1 Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>										

Step	Action																				
<b>Step 15. Amplifier Hybridization</b>  20 min	<p><b>A.</b> Using the table below as a guide, prepare the Working Amp1 Solution by diluting Amp1 QF, 1:100 in prewarmed Amplifier Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table><tr><th colspan="2">Working Amp1 Solution per Slide</th></tr><tr><th>Reagent</th><th>Volume</th></tr><tr><td>Amplifier Diluent QF (prewarmed to 40 °C)</td><td>396 μL</td></tr><tr><td>Amp1 QF</td><td>4 μL</td></tr><tr><td>Total volume</td><td>400 μL</td></tr></table> <p><b>B.</b> Remove each slide from Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench and immediately add 400 μL of Working Amp1 Solution to each tissue section.</p> <p><b>C.</b> Place slides in the ThermoBrite Incubator. Close the lid and incubate at 40 ± 1 °C for 15 min.</p> <p><b>IMPORTANT: Incubation time should not exceed 15 min.</b></p>	Working Amp1 Solution per Slide		Reagent	Volume	Amplifier Diluent QF (prewarmed to 40 °C)	396 μL	Amp1 QF	4 μL	Total volume	400 μL										
Working Amp1 Solution per Slide																					
Reagent	Volume																				
Amplifier Diluent QF (prewarmed to 40 °C)	396 μL																				
Amp1 QF	4 μL																				
Total volume	400 μL																				
<b>Step 16. Wash Slides</b>  10 min	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Amp1 Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>																				
<b>Step 17. Label Probe-AP Hybridization</b>  25 min	<p><b>A.</b> Using the table below (left side) as a guide, prepare 1:10 Working Label Probe-AP Solution by diluting Label Probe-AP 1:10 in prewarmed Label Probe Diluent QF and briefly vortexing to mix.</p> <p><b>B.</b> Using the table below (right side) as a guide, prepare Working Label Probe-AP Solution by diluting the 1:10 Working Label Probe-AP Solution to 1:1000 in prewarmed Label Probe Diluent QF and briefly vortexing to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table><tr><th colspan="2">1:10 Working Label Probe-AP Solution per Slide</th><th colspan="2">1:1000 Working Label Probe-AP Solution per Slide</th></tr><tr><th>Reagent</th><th>Volume</th><th>Reagent</th><th>Volume</th></tr><tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>54 μL</td><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>396 μL</td></tr><tr><td>Label Probe-AP</td><td>6 μL</td><td>1:10 Working Label Probe-AP (from 1:10 dilution)</td><td>4 μL</td></tr><tr><td>Total volume</td><td>60 μL</td><td></td><td>400 μL</td></tr></table> <p><b>C.</b> Discard any remainder of 1:10 Working Label Probe-AP solution.</p> <p><b>D.</b> Remove each slide from the Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench and immediately add 400 μL of 1:1000 Working Label Probe-AP Solution to each tissue section.</p> <p><b>E.</b> Place the slides in the ThermoBrite Incubator. Close the lid and incubate at 40 ± 1 °C for 15 min.</p> <p><b>IMPORTANT: Incubation time should not exceed 15 min.</b></p>	1:10 Working Label Probe-AP Solution per Slide		1:1000 Working Label Probe-AP Solution per Slide		Reagent	Volume	Reagent	Volume	Label Probe Diluent QF (prewarmed to 40 °C)	54 μL	Label Probe Diluent QF (prewarmed to 40 °C)	396 μL	Label Probe-AP	6 μL	1:10 Working Label Probe-AP (from 1:10 dilution)	4 μL	Total volume	60 μL		400 μL
1:10 Working Label Probe-AP Solution per Slide		1:1000 Working Label Probe-AP Solution per Slide																			
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Label Probe-AP	6 μL	1:10 Working Label Probe-AP (from 1:10 dilution)	4 μL																		
Total volume	60 μL		400 μL																		

Step	Action
<b>Step 18. Wash Slides</b>  <b>13 min</b>	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Label Probe-AP Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 3 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 3 min with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>
<b>Step 19. Apply Fast Red Substrate</b>  <b>1 hr</b>	<p><b>A.</b> Remove each slide from the Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench.</p> <p><b>B.</b> Immediately add 400 <math>\mu</math>L of the AP-Enhancer Solution to each tissue section (pipet directly from bottle) and incubate at RT for 5 min. while preparing the Fast Red Substrate.</p> <p><b>C.</b> Prepare the Fast Red Substrate: in a 15 ml conical tube, add 5 ml of Naphthol Buffer and one Fast Red Tablet. Vortex at high speed to completely dissolve the tablet.</p> <p><b>D.</b> Decant the AP Enhancer Solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Immediately add 400 <math>\mu</math>L of Fast Red Substrate onto each tissue section.</p> <p><b>E.</b> Place the slides in the ThermoBrite Incubator. Close the lid and incubate at <math>40 \pm 1</math> °C for 30 min.</p> <p><b>F.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.</p> <p><b>G.</b> After incubation, decant the Fast Red Substrate from the slides and insert them into the slide rack.</p> <p><b>H.</b> Move the slide rack up and down several times for 1 min to rinse off the Fast Red Substrate.</p> <p><b>I.</b> Retrieve 200 mL of 4% formaldehyde (used previously) and pour in the clear staining dish labeled for formaldehyde.</p> <p><b>J.</b> Move the slide rack to the clear staining dish containing 200 mL of 4% formaldehyde and incubate for 5 min under a fume hood.</p> <p><b>K.</b> Rinse off the residual formaldehyde by transferring the slide rack to a clear staining dish containing fresh 1X PBS. Move the slide rack up and down several times for 1 min.</p>
<b>Step 20. Counterstain</b>  <b>50 min</b>	<p><b>A.</b> Remove each slide from 1X PBS and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench and add 500 <math>\mu</math>L of Gill's Hematoxylin Solution. Incubate the slides for 5 min at RT.</p> <p><b>B.</b> Insert the empty slide rack into a clear staining dish containing ddH<sub>2</sub>O.</p> <p><b>C.</b> After incubation, decant Gill's Hematoxylin Solution from the slides and insert them into the slide rack containing ddH<sub>2</sub>O. Move the slide rack up and down several times to rinse off Gill's Hematoxylin Solution.</p> <p><b>D.</b> Decant the ddH<sub>2</sub>O, refill with 200 mL fresh ddH<sub>2</sub>O and move slide rack up and down several times. Repeat this step one more time.</p> <p><b>E.</b> Decant the ddH<sub>2</sub>O, refill with 200 mL 0.01% ammonium hydroxide and incubate for 10 sec.</p> <p><b>F.</b> Decant 0.01% ammonium hydroxide, refill with fresh ddH<sub>2</sub>O and move slide rack up and down several times.</p> <p><b>G.</b> <i>Optional.</i> If you plan to view slides using the fluorescent microscope, then move slide rack into a clear staining dish containing 200 mL DAPI staining solution. Incubate the slides for 1 min. Decant DAPI staining solution, refill with 200 mL fresh ddH<sub>2</sub>O and move the slide rack up and down several times to rinse off DAPI solution. DAPI staining step must be performed after the Gill's Hematoxylin staining step.</p> <p><b>H.</b> Remove the slides from the slide rack and decant the ddH<sub>2</sub>O by flicking and briefly placing the slide on its edge on a laboratory wipe. Place them onto a paper towel to air dry.</p> <p><b>I.</b> Ensure that slide sections are completely dry before mounting (about 20 min).</p>



Step	Action
<p><b>Step 21. Add Coverslip and Image</b></p> <p>20 min</p>	<p><b>A.</b> Add a minimum of two drops of DAKO Ultra Mount mounting medium to tissue section without making any bubbles. Cover the slide section with a piece of 24 mm x 55 mm cover glass.</p> <p><b>B.</b> Dab off the excess mounting medium and image the result under a bright field microscope and/or fluorescent microscope.</p> <hr/> <p><b>IMPORTANT:</b> Slides should be viewed and imaged within one week of mounting to ensure the accuracy and validity of the results. Bubbles may start to form after one week making imaging difficult. Please refer to <a href="#">Remounting Slides After Bubbles Form on page 55</a> for the remounting procedure.</p> <hr/> <p><b>NOTE:</b> Fluorescence signals will fade over time. However, chromogenic signal will remain stable for at least 3 months.</p> <hr/> <p><b>C.</b> 0.01% ammonium hydroxide solution can be stored at RT for up to one month. Discard all remaining reagent and solution preparations.</p>



## Assessing the Results

### Overview

This section provides examples of data taken from the QuantiGene ViewRNA ISH Tissue Assay performed on rat kidney tissue examining Arbp RNA expression. Use these examples as a gauge for your specific target and sample type.

### Tissue Morphology and Signal Strength

Table 5.2 Examples of different cell morphologies and signal strength

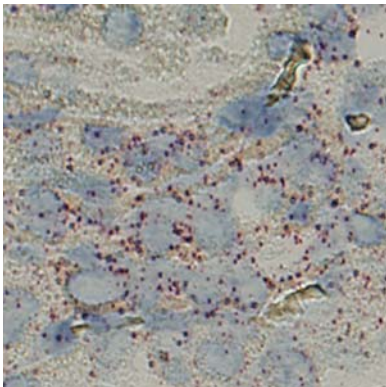
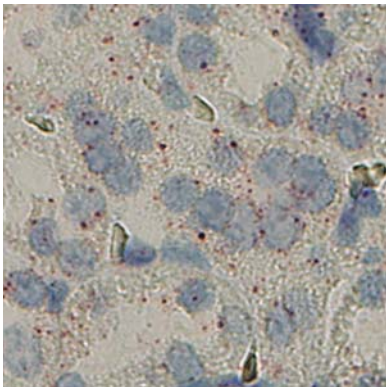
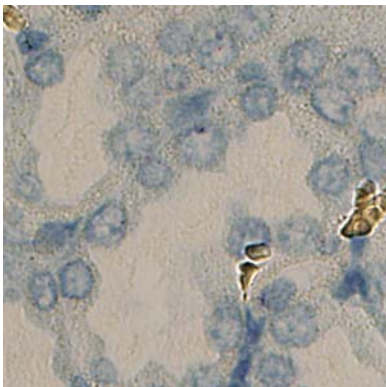
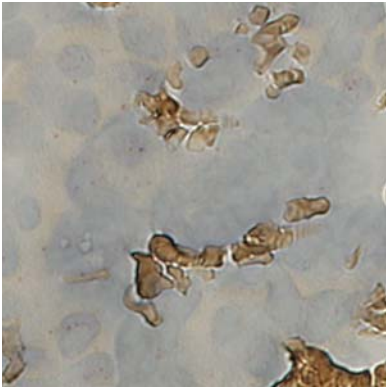
Example	Bright Field
<b>Strong Signal/Optimal Morphology</b> Good nuclei definition, cells are easily distinguished, clearly visible, punctuated red dots.	
<b>Weaker Signal</b> Faint red dots, diffused dots, good morphology. Refer to the troubleshooting section for more information.	
<b>Negative Control</b> Less than 1 dot/10 cells. Good morphology and good staining of nuclei is required.	

Table 5.2 Examples of different cell morphologies and signal strength

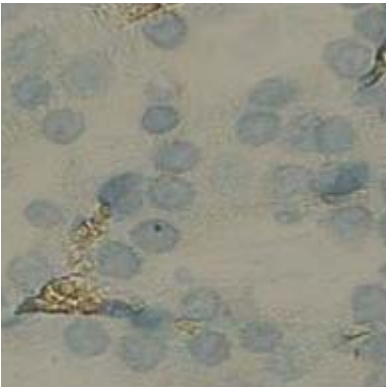
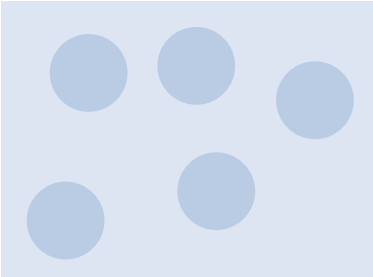
Example	Bright Field
<b>Poor Morphology</b> Treatment conditions not optimal. Cell membrane is over digested.	

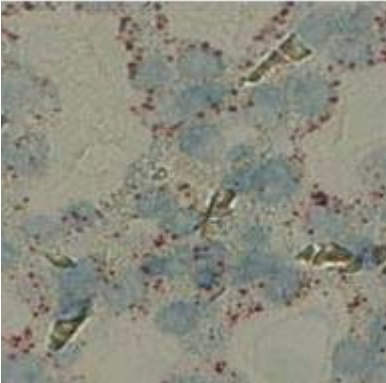
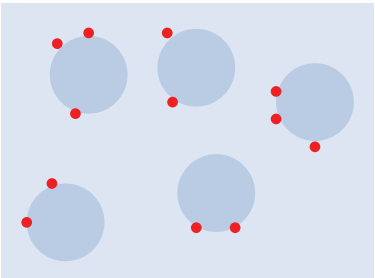
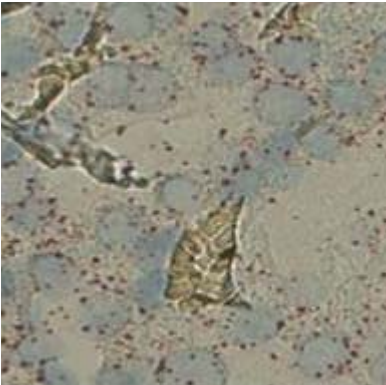
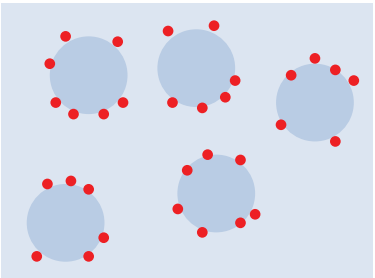
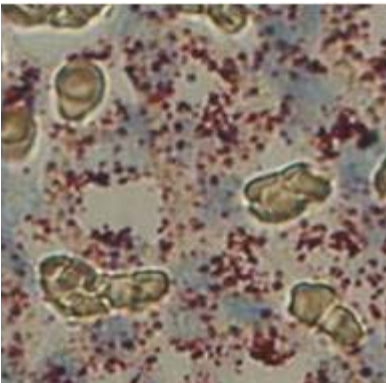
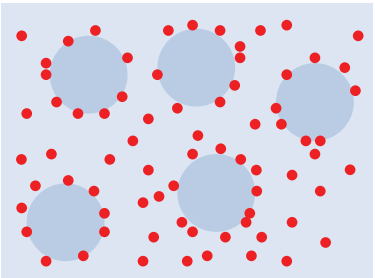
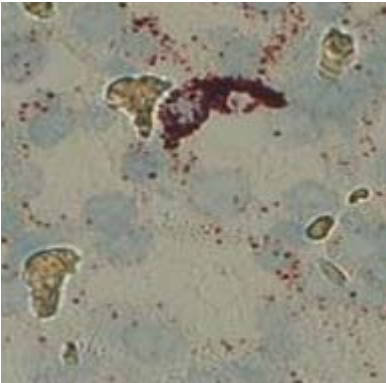
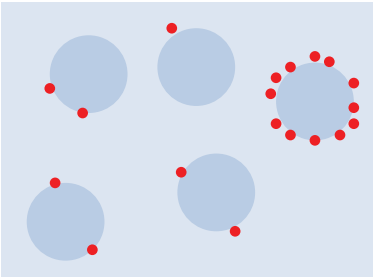
Quantifying Results

Table 5.3 How to quantify results

Step	Action
1	Examine all areas of the tissue and select an area that is a good representation of the entire slide.
2	Select an area where you can observe a minimum of 20 cells.
3	Count the number of Fast Red dots surrounding the cells.
4	Take the number of Fast Red dots and divide it by the number of cells counted to obtain a dot-per-cell count.

Examples of Quantification

Treatment	Bright Field	Five Representative Cells
<b>Negative Control</b> Use as a reference to measure background for non-specific binding and to compare cell morphology.		

Treatment	Bright Field	Five Representative Cells
<p><b>Clu</b></p> <p>A low-expresser gene. On rat kidney, a typical expression level is greater than 2 dots/cell.</p> <p><b>Quantitation</b></p> <p>Cells (blue) = 5, Fast Red dots (red) = 12, Dots/cell = 2.4</p>		
<p><b>Arbp</b></p> <p>A medium-expresser gene. In rat kidney, typical expression level is greater than 6 dots/cell.</p> <p><b>Quantitation</b></p> <p>Cells (blue) = 5, Fast Red dots (red) = 32, Dots/cell = 6.4</p>		
<p><b>Ubc</b></p> <p>A high-expresser gene. In rat kidney, UBC is typically saturated.</p> <p><b>Quantitation</b></p> <p>Cells (blue) = 5, Fast Red dots (red) = Saturation, Dots/cell = Saturation</p>		
<p><b>Localization</b></p> <p>Fast Red substrate is expressed predominantly in one area.</p>		



## Troubleshooting

### Weak or No Signals

Table 6.1 Troubleshooting Weak or No Signal

Probable Cause	Recommended Action
Tissue preparation	Repeat pretreatment assay optimization procedure to determine optimal boiling time and protease digestion time.
Tissue dries up during hybridization steps	<p>ThermoBrite Incubator recommendations:</p> <ul style="list-style-type: none"> <li>■ Prewet the humidifying strips inside the ThermoBrite before starting hybridization</li> <li>■ Make sure the ThermoBrite is placed on a leveled bench.</li> <li>■ Calibrate the ThermoBrite at 40°C using QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).</li> <li>■ Make sure to close the ThermoBrite lid during hybridization steps.</li> <li>■ Process one slide at a time to prevent drying after tissue pretreatment.</li> </ul> <p>Prevent sections from drying out by:</p> <ul style="list-style-type: none"> <li>■ Preparing enough reagents and use the recommended volumes for each step of the assay.</li> <li>■ Ensuring that you have a solid seal when drawing your hydrophobic barriers.</li> <li>■ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite (except protease digestion step).</li> </ul>
Tissue dries up during processing	<p>Keep tissue section moist starting from the pretreatment boiling step by:</p> <ul style="list-style-type: none"> <li>■ Adding respective reagents immediately after decanting solution from the slides.</li> <li>■ Limiting tissue exposure to air for too long before adding hybridization reagents.</li> <li>■ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite.</li> </ul>
Tissue over-fixed after protease digestion	Make sure the tissue sections are not fixed for more than 5 min. in 4% formaldehyde after protease digestion.
Reagents applied in wrong sequence	Apply target Probe Set, PreAmp1, Amp1, Label Probe-AP in the correct order.
Incorrect storage condition	Store the components at the suggested storage condition as written on the component label or kit boxes.
Hybridization temperature not optimal	Calibrate the ThermoBrite at 40°C using a QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
Mounting solution contained alcohol	Use recommended UltraMount to mount your tissue. Avoid any mounting solution containing alcohol.
Improper fixation	Make sure correct concentration of 10% formaldehyde was used to fix the slides in respective steps.
Fast Red solution not freshly prepared	Prepare Fast Red solution immediately before use.
Gene of interest not expressed	Check positive control

## High Background

**Table 6.2** Troubleshooting High Background

Probable Cause	Recommended Action
Tissue dries up during processing	ThermoBrite Incubator recommendations: <ul style="list-style-type: none"> <li>■ Prewet the humidifying strips inside the ThermoBrite before starting hybridization</li> <li>■ Make sure the ThermoBrite is placed on a leveled bench.</li> <li>■ Calibrate the ThermoBrite at 40°C using QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).</li> <li>■ Make sure to close the ThermoBrite lid during hybridization steps.</li> <li>■ Process one slide at a time to prevent drying after tissue pretreatment.</li> </ul> Prevent sections from drying out by: <ul style="list-style-type: none"> <li>■ Preparing enough reagents and use the recommended volumes for each step of the assay.</li> <li>■ Ensuring that you have a solid seal when drawing your hydrophobic barriers.</li> <li>■ Adding all working reagents onto the slides before moving them to the 40°C ThermoBrite (except protease digestion step).</li> </ul>
Incomplete removal of paraffin	<ul style="list-style-type: none"> <li>■ Use fresh HistoClear solution.</li> <li>■ Immediately submerge the warm slides into the HistoClear solution after 80 °C baking.</li> </ul>
Boiling and protease digestion time not optimal	Perform the pretreatment assay optimization procedure to determine correct conditions for your tissue.
Insufficient washing	<ul style="list-style-type: none"> <li>■ Move the slide rack up and down with frequent agitation.</li> <li>■ Increase wash incubation time by 1 min. per wash.</li> </ul>
Concentration of hybridization reagents was too high	Double check the dilution calculations for all working solutions.

## Background Examples

The following figures illustrate some of the different types of background problems that might occur with the assay.

**Table 6.3** Examples of different backgrounds

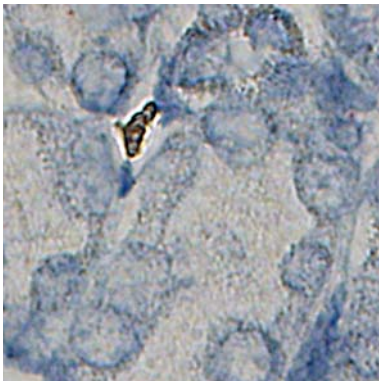
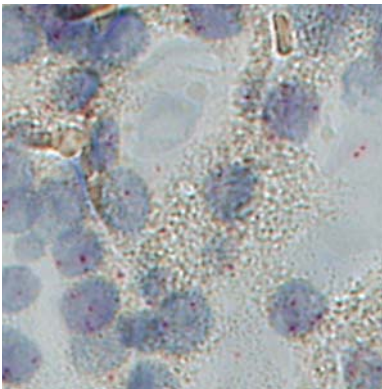
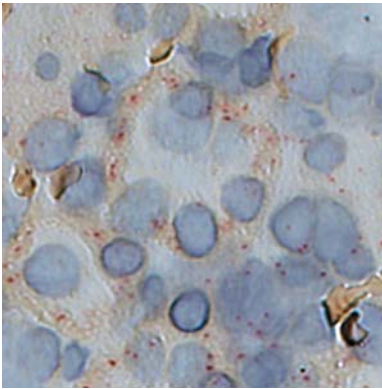
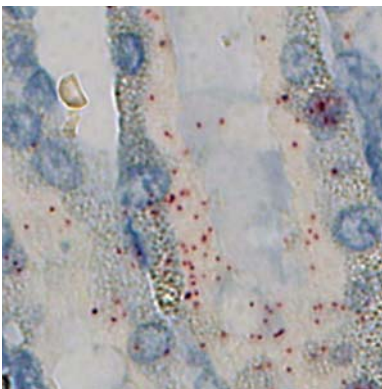
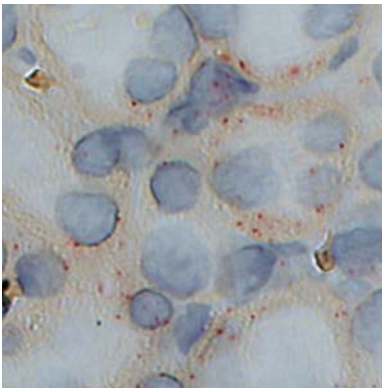
Example	Bright Field
<b>Clean Background</b> The first example shows a clean background. There is less than 1 dot/10 cells.	



Table 6.3 Examples of different backgrounds

Example	Bright Field
<p><b>High Background due to Contamination</b>            Very strong, sharp dots are observed. In this case the cause was Probe Set Diluent QF contaminated with target Probe Set.</p>	
<p><b>Diffused Background due to Dried-Up Tissue</b>            Faint dots and diffusion are observed. In this case, the tissue section dried up during processing.</p>	
<p><b>High Background in Nuclei due to Excess Pretreatment or High Concentration of Reagents</b>            Dots appear inside nuclei. In this case, the tissue was boiled and protease treated too long during pretreatment and/or PreAmp1 QF or Amp1 QF reagent concentration was too high.</p>	
<p><b>High Background in Extracellular Matrix due to Incomplete Paraffin Removal</b>            Dots appear in extracellular matrix. In this case, the paraffin was not completely removed.</p>	



## Pretreatment Assay Optimization Procedures and Typical Results

### About the Pretreatment Optimization and Typical Results

The QuantiGene ViewRNA ISH Tissue Assay can be run in a single long day or broken up over 2 days for added flexibility. There is an optional assay stop point, following the target probe hybridization, where the assay can be stopped and work can be continued the following day.

For convenience, the procedure is sectioned into two parts:

- Part 1: Sample Preparation and Target Probe Set Hybridization
- Part 2: Signal Amplification and Detection

with the stop point at the end of Part 1. We do not recommend stopping at any other point in the assay.

The two conditions to be optimized, tissue pretreatment boiling time and protease digestion time are included in Part 1: Sample Preparation.

In this appendix, we provide two procedures for the recommended pretreatment assay optimization, based on equipment used, ThermoBrite Incubator or dry oven and tissue culture incubator without CO<sub>2</sub>. For more information about pretreatment assay optimization, see [Experimental Design Guidelines on page 7](#).

Also provided in this appendix are typical results for both bright field and fluorescent detection.

### Optimization Procedure Overview

You will need to prepare eleven, 4-6 µm thick FFPE tissue sections from a block, or blocks which were prepared in the same way (fixation time, section thickness, and tissue type) as the FFPE tissue of your interest. Each slide will be treated with a different set of conditions and hybridized with a control target Probe Set of medium expression. This control target has characteristics similar to a traditional housekeeping target and should have consistent homogenous expression in your samples. Once an optimal condition is determined for your sample type, apply those conditions to your targets of interest.

### Optimization Experimental Design Layout

Table A.1 Optimization Experiment Setup

Protease Incubation Time (min)	Pretreatment Boiling Time (min)			
	0	5	10	20
0	Slide 1 with probe			
10		Slide 2 with Probe	Slide 5 with probe	Slide 9 with probe
20		Slide 3 with probe	Slide 6 with probe Slide 7 with no probe	Slide 10 with probe
40		Slide 4 with probe	Slide 8 with probe	Slide 11 with probe

### Important Procedural Notes and Guidelines


- Perform all steps in a well-ventilated area at room temperature unless otherwise noted.
- Procedure assumes running a maximum of 12 slides at a time.

- Maximum tissue area for this procedure is 30 mm x 22 mm.
- The QuantiGene ViewRNA ISH Tissue Assay is optimized for chromogenic visualization under bright field microscope. Detection using a fluorescent microscope may require additional optimization.
- Do not mix and match kit components from different lots.
- Use a pencil to label all slides.
- Always use freshly prepared solutions in the Tissue Tek Staining and Clearing Agent dishes. Do not save or reuse solutions unless directed to do so (for example with the 4% formaldehyde solution).
- The Tissue Tek Staining Dish (clear) will be referred to as the “clear staining dish” throughout the assay procedure.
- The Tissue Tek Clearing Agent Dish (green) will be referred to as the “green clearing agent dish” throughout the assay procedure.
- Throughout the procedure, dedicate one clear staining dish for fixing in formaldehyde (we recommend labeling this dish). The other two clear staining dishes can be used interchangeably for: 1X PBS, 95% Ethanol, Wash Buffer and Storage Buffer. Rinse staining dishes in between steps with ddH<sub>2</sub>O.
- The tissue culture incubation without CO<sub>2</sub> will be referred to as a humidified incubator throughout the assay procedure.
- Validate the temperature of the ThermoBrite Incubator or dry oven using the QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
- Validate the temperature and humidity (should be  $\geq 65\%$ ) of the humidified incubator with a temperature/humidity meter (Fisher Scientific P/N 11-661-19).
- Unless otherwise stated, all reagents prepared should be stored at room temperature until use.
- Typical processing times included in the assay procedure assume that preparation for the following step is being done during the incubation periods.
- Before opening reagents supplied in the microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- Discard all reagents in accordance with local, state, and federal laws.

## Part 1: Sample Preparation and Target Probe Hybridization

### Part 1 Procedure, if using a ThermoBrite Incubator

Step	Action
<b>Step 1. Bake Slides</b>  35 min	<p><b>A.</b> Use a pencil to label the slides.</p> <p><b>B.</b> Set ThermoBrite Incubator at <math>60 \pm 1^\circ\text{C}</math> and bake the slides for 30 min with the lid open.</p> <hr/> <p><b>NOTE:</b> This increases tissue attachment to the slide.</p> <hr/>
<b>Step 2. Prepare Buffers and Reagents While Slides Bake</b>  0 min	<p><b>A.</b> Prepare 2 L of 1X PBS: To a 2 L container add 200 mL of 10X PBS and 1.8 L ddH<sub>2</sub>O.</p> <p><b>B.</b> Prepare 10% formaldehyde in 1X PBS under a fume hood:</p> <hr/> <p><b>WARNING:</b> Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.</p> <hr/> <p>To a 200 mL capacity container add 146 ml 1X PBS and 54 mL of 37% formaldehyde and mix well.</p> <p><b>C.</b> Prepare 4% formaldehyde in 1X PBS: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well.</p> <p><b>D.</b> Prepare 3 L of Wash Buffer: To a 3 L capacity container add components in the following order to prevent precipitation from forming and then mix well: 2.5 L ddH<sub>2</sub>O, 27 mL Wash Comp 1, 7.5 mL Wash Comp 2, and ddH<sub>2</sub>O to 3 L.</p> <p><b>E.</b> Ensure availability of:</p> <ul style="list-style-type: none"> <li>■ 400 mL 95% ethanol</li> <li>■ 400 mL ddH<sub>2</sub>O</li> <li>■ 200 mL HistoClear</li> </ul> <p><b>F.</b> Pour solutions into Tissue Tek dishes:</p> <ul style="list-style-type: none"> <li>■ In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish</li> <li>■ In a fume hood, pour 200 mL of HistoClear into green Clearing Agent Dish</li> <li>■ Pour 200 mL of 95% ethanol into clear staining dish</li> <li>■ Pour 200 mL of 1X PBS into clear staining dish</li> </ul> <p><b>G.</b> Prewarm 40 mL of 1X PBS and Probe Set Diluent QF to <math>40 \pm 1^\circ\text{C}</math>.</p> <p><b>H.</b> Thaw Probe Set(s). Place on ice until use.</p> <p><b>I.</b> Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH<sub>2</sub>O.</p> <p><b>J.</b> Prepare 500 mL of Storage Buffer (for Optional Stop Point): To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH<sub>2</sub>O and mix well.</p>
<b>Step 3. Fix Slides</b>  1 hr 5 min	<p><b>A.</b> Insert slides into an empty slide rack and submerge into a clear staining dish containing 10% formaldehyde. Incubate for 1 hour at room temperature (RT) under a fume hood.</p> <p><b>B.</b> Remove the slide rack from the 10% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min. with frequent agitation.</p> <p><b>C.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min. with frequent agitation.</p> <p><b>D.</b> Remove each slide and decant the 1X PBS by flicking and placing it on its edge on a laboratory wipe. Place the slides on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.</p> <p><b>E.</b> Set ThermoBrite Incubator to <math>80^\circ\text{C}</math>.</p>

Step	Action
<b>Step 4. Deparaffinization</b>  <b>30 min</b>	<p><b>A.</b> Insert an empty slide rack into a green clearing agent dish containing 200 mL HistoClear solution. Cover the dish with the lid and bring it next to the ThermoBrite Incubator.</p> <p><b>B.</b> Bake the slides on the ThermoBrite Incubator with the lid open at <math>80 \pm 1</math> °C for 3 min. The paraffin should melt as soon as the slides are on the ThermoBrite Incubator.</p> <p><b>C.</b> Remove lid from green Tissue Tek dish, and immediately insert the warm slides in the HistoClear solution, cover the dish with the lid and incubate under a fume hood at RT for 10 min. with frequent agitation.</p> <p><b>D.</b> Lift the slide rack from the HistoClear solution and submerge into the clear staining dish containing 200 mL 95% ethanol and incubate for 1 min. with frequent agitation.</p> <p><b>E.</b> Decant the 95% ethanol, refill with 200 mL of fresh 95% ethanol, and incubate for 1 min. with frequent agitation.</p> <p><b>F.</b> Remove the slides from the slide rack and place them on a paper towel to air dry for 5 min at RT. Discard the 95% ethanol.</p>
<b>Step 5. Draw Hydrophobic Barrier</b>  <b>1 hr</b>	<p><b>A.</b> Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.</p> <p><b>B.</b> To create a hydrophobic barrier, place the slide over the template image below, tissue sections should fall inside blue rectangle, and lightly trace the blue rectangle 2 to 4 times with the ImmEdge Hydrophobic Barrier Pen. It may be necessary to draw the hydrophobic barrier over tissue edges for larger sections or sections mounted close to the edge of the slide. Allow for barrier to dry at RT for 20-30 min.</p> <hr/> <p><b>IMPORTANT: Consistently draw hydrophobic barrier size as indicated in template, even if using smaller tissue sections.</b></p> <hr/> <p><b>IMPORTANT: Draw the barrier 2-4 times to ensure a solid seal.</b></p> <hr/> 
<b>Step 6. Tissue Pretreatment</b>  <b>25 min</b>	<p><b>A.</b> Bring 500 mL of 1X Pretreatment Solution to boil (100 °C) in a 1 L beaker tightly covered with aluminum foil on a hot plate. When boiling is reached, use a water-proof probe thermometer to measure and maintain the boiling temperature at 95-100 °C.</p> <p><b>B.</b> Set slide 1 aside on the lab bench.</p> <p><b>C.</b> Load slides 9, 10, and 11 into the slide rack.</p> <p><b>D.</b> Using a pair of forceps, submerge the slide rack into the boiling 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate for 10 min at 95-100 °C.</p> <p><b>E.</b> At the end of 10 min, use forceps to add slides 5, 6, 7, and 8 into the boiling 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min.</p> <p><b>F.</b> At the end of 5 min, use forceps to add slides 2, 3, and 4 into the boiling 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min.</p> <p><b>G.</b> Using a pair of forceps, remove the slide rack loaded with slides and submerge it into a clear staining dish containing 200 mL ddH<sub>2</sub>O. Incubate for 1 min with frequent agitation.</p> <p><b>H.</b> Decant the ddH<sub>2</sub>O and refill the clear staining dish with fresh ddH<sub>2</sub>O. Incubate for 1 min. with frequent agitation.</p> <p><b>I.</b> Transfer the slide rack to a clear staining dish containing 1X PBS.</p> <hr/> <p><b>IMPORTANT: From this point forward do not let the tissue sections dry out.</b></p> <hr/>


Step	Action										
<b>Step 7. Protease Digestion and Fixation</b>  50 min	<p><b>A.</b> Set the ThermoBrite Incubator to 40 °C and insert two wet humidifying strips.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working Protease Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Protease QF</td><td>4 µL</td></tr> <tr> <td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Leave slide 1 on the lab bench as it is excluded from this step.</p> <p><b>D.</b> Remove slides 4, 8, and 11 from the slide rack, flick off excess 1X PBS and place them on a paper towel. Leave remaining slides in 1X PBS until appropriate incubation time.</p> <p><b>E.</b> Add 500 µL of prewarmed 1X PBS to slides 4, 8, and 11 and incubate on the ThermoBrite Incubator with the lid open for 3 min. at 40 ± 1 °C.</p> <p><b>F.</b> Working with one slide at a time, decant the 1X PBS, place the slide onto the ThermoBrite Incubator, and then add 400 µL of the Working Protease Solution onto the tissue section. Repeat for the rest of the slides.</p> <hr/> <p><b>IMPORTANT: Process ONE slide at a time to avoid drying.</b></p> <hr/> <p><b>G.</b> Close the lid and incubate for 19 min at 40 ± 1 °C.</p> <p><b>H.</b> Immediately remove slides 3, 6, 7, and 10 from the slide rack, flick off excess 1X PBS and place them on a paper towel.</p> <p><b>I.</b> At the end of 19 min, add 500 µL prewarmed 1X PBS to slides 3, 6, 7, and 10, open the lid and place slides onto the ThermoBrite Incubator.</p> <p><b>J.</b> Close the lid and incubate for 3 min.</p> <p><b>K.</b> At the end of 3 min, decant the 1X PBS from slides 3, 6, 7, and 10 and add 400 µL of Working Protease Solution on to the tissue section. Close the lid and incubate for 5 min.</p> <p><b>L.</b> Immediately remove slides 2, 5, and 9 from the slide rack, flick off excess 1X PBS and place them on a paper towel. Leave remaining slides in 1X PBS until appropriate incubation time.</p> <p><b>M.</b> At the end of 5 min add 500 µL prewarmed 1X PBS to slides 2, 5, and 9, open the lid and place slides onto the ThermoBrite Incubator. Close lid and incubate for 3 min.</p> <p><b>N.</b> At the end of 3 min, decant the 1X PBS from slides 2, 5, and 9 and add 400 µL of Working Protease Solution onto the tissue section. Close lid and incubate for 10 min.</p> <p><b>O.</b> At the end of 10 min (40 min total incubation time), decant the Working Protease Solution from all slides, insert them into the slide rack and transfer slide rack into clear staining dish with 200 mL 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>P.</b> Retrieve slide 1 and add to slide rack in PBS. There should be 11 slides in the slide rack.</p> <p><b>Q.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>R.</b> Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.</p> <p><b>S.</b> Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.</p> <p><b>T.</b> Transfer the slide rack from the 4% formaldehyde solution to the clear staining dish containing 1X PBS, and incubate for 1 min. with frequent agitation.</p> <p><b>U.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>V.</b> Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.</p> <p><b>W.</b> Proceed to <a href="#">Step 8. Target Probe Set Hybridization on page 17</a> to continue the assay procedure.</p>	Working Protease Solution per Slide		Reagent	Volume	Protease QF	4 µL	1X PBS (prewarmed to 40 °C)	396 µL	Total volume	400 µL
Working Protease Solution per Slide											
Reagent	Volume										
Protease QF	4 µL										
1X PBS (prewarmed to 40 °C)	396 µL										
Total volume	400 µL										

## Part 1: Sample Preparation and Target Probe Hybridization

### Part 1 Procedure, if using a dry oven and humidified incubator

Step	Action
<b>Step 1. Bake Slides</b>  <b>35 min</b>	<p><b>A.</b> Use a pencil to label the slides.</p> <p><b>B.</b> Set dry oven at <math>60 \pm 1^\circ\text{C}</math>, insert slides into slide rack, and bake the slides for 30 min.</p> <hr/> <p><b>NOTE:</b> This increases tissue attachment to the slide.</p> <hr/>
<b>Step 2. Prepare Buffers and Reagents While Slides Bake</b>  <b>0 min</b>	<p><b>A.</b> Prepare 2 L of 1X PBS: To a 2 L container add 200 mL of 10X PBS and 1.8 L ddH<sub>2</sub>O.</p> <p><b>B.</b> Prepare 10% formaldehyde in 1X PBS under a fume hood:</p> <hr/> <p><b>WARNING:</b> Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.</p> <hr/> <p>To a 200 mL capacity container add 146 ml 1X PBS and 54 mL of 37% formaldehyde and mix well.</p> <p><b>C.</b> Prepare 4% formaldehyde in 1X PBS: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well.</p> <p><b>D.</b> Prepare 3 L of Wash Buffer: To a 3 L capacity container add components in the following order to prevent precipitation from forming and then mix well: 2.5 L ddH<sub>2</sub>O, 27 mL Wash Comp 1, 7.5 mL Wash Comp 2, and ddH<sub>2</sub>O to 3 L.</p> <p><b>E.</b> Ensure availability of:</p> <ul style="list-style-type: none"> <li>■ 400 mL 95% ethanol</li> <li>■ 400 mL ddH<sub>2</sub>O</li> <li>■ 200 mL HistoClear</li> </ul> <p><b>F.</b> Pour solutions into Tissue Tek dishes:</p> <ul style="list-style-type: none"> <li>■ In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish</li> <li>■ In a fume hood, pour 200 mL of HistoClear into green Clearing Agent Dish</li> <li>■ Pour 200 mL of 95% ethanol into clear staining dish</li> <li>■ Pour 200 mL of 1X PBS into clear staining dish</li> </ul> <p><b>G.</b> Prewarm 240 mL of 1X PBS and Probe Set Diluent QF to <math>40 \pm 1^\circ\text{C}</math>.</p> <p><b>H.</b> Thaw Probe Set(s). Place on ice until use.</p> <p><b>I.</b> Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH<sub>2</sub>O.</p> <p><b>J.</b> Prepare 500 mL of Storage Buffer (for Optional Stop Point): To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH<sub>2</sub>O and mix well.</p>
<b>Step 3. Fix Slides</b>  <b>1 hr 5 min</b>	<p><b>A.</b> Insert slides into an empty slide rack and submerge into a clear staining dish containing 10% formaldehyde. Incubate for 1 hour at room temperature (RT) under a fume hood.</p> <p><b>B.</b> Remove the slide rack from the 10% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min. with frequent agitation.</p> <p><b>C.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min. with frequent agitation.</p> <p><b>D.</b> Remove each slide and decant the 1X PBS by flicking and placing it on its edge on a laboratory wipe. Place the slides on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.</p> <p><b>E.</b> Set dry oven to <math>80^\circ\text{C}</math>.</p>



Step	Action
<b>Step 4. Deparaffinization</b>  <b>30 min</b>	<p><b>A.</b> Insert an empty slide rack into a green clearing agent dish containing 200 mL HistoClear solution. Cover the dish with the lid and bring it next to the dry oven.</p> <p><b>B.</b> Bake the slides in the dry oven at <math>80 \pm 1^\circ\text{C}</math> for 3 min. The paraffin should melt as soon as the slides are in the dry oven.</p> <p><b>C.</b> Remove lid from green Tissue Tek dish, and immediately insert the warm slides in the HistoClear solution, cover the dish with the lid and incubate under a fume hood at RT for 10 min. with frequent agitation.</p> <p><b>D.</b> Lift the slide rack from the HistoClear solution and submerge into the clear staining dish containing 200 mL 95% ethanol and incubate for 1 min. with frequent agitation.</p> <p><b>E.</b> Decant the 95% ethanol, refill with 200 mL of fresh 95% ethanol, and incubate for 1 min. with frequent agitation.</p> <p><b>F.</b> Remove the slides from the slide rack and place them on a paper towel to air dry for 5 min at RT. Discard the 95% ethanol.</p>
<b>Step 5. Draw Hydrophobic Barrier</b>  <b>1 hr</b>	<p><b>A.</b> Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.</p> <p><b>B.</b> To create a hydrophobic barrier, place the slide over the template image below, tissue sections should fall inside blue rectangle, and lightly trace the blue rectangle 2 to 4 times with the ImmEdge Hydrophobic Barrier Pen. It may be necessary to draw the hydrophobic barrier over tissue edges for larger sections or sections mounted close to the edge of the slide. Allow for barrier to dry at RT for 20-30 min.</p> <hr/> <p><b>IMPORTANT: Consistently draw hydrophobic barrier size as indicated in template, even if using smaller tissue sections.</b></p> <hr/> <p><b>IMPORTANT: Draw the barrier 2-4 times to ensure a solid seal.</b></p> <hr/> 
<b>Step 6. Tissue Pretreatment</b>  <b>25 min</b>	<p><b>A.</b> Bring 500 mL of 1X Pretreatment Solution to boil (<math>100^\circ\text{C}</math>) in a 1 L beaker tightly covered with aluminum foil on a hot plate. When boiling is reached, use a water-proof probe thermometer to measure and maintain the boiling temperature at <math>95\text{--}100^\circ\text{C}</math>.</p> <p><b>B.</b> Set slide 1 aside on the lab bench.</p> <p><b>C.</b> Load slides 9, 10, and 11 into the slide rack.</p> <p><b>D.</b> Using a pair of forceps, submerge the slide rack into the boiling 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate for 10 min at <math>95\text{--}100^\circ\text{C}</math>.</p> <p><b>E.</b> At the end of 10 min, use forceps to add slides 5, 6, 7, and 8 into the boiling 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min.</p> <p><b>F.</b> At the end of 5 min, use forceps to add slides 2, 3, and 4 into the boiling 1X Pretreatment Solution. Re-cover the glass beaker with aluminum foil and incubate for 5 min.</p> <p><b>G.</b> Using a pair of forceps, remove the slide rack loaded with slides and submerge it into a clear staining dish containing 200 mL <math>\text{ddH}_2\text{O}</math>. Incubate for 1 min with frequent agitation.</p> <p><b>H.</b> Decant the <math>\text{ddH}_2\text{O}</math> and refill the clear staining dish with fresh <math>\text{ddH}_2\text{O}</math>. Incubate for 1 min. with frequent agitation.</p> <p><b>I.</b> Transfer the slide rack to a clear staining dish containing 1X PBS.</p> <hr/> <p><b>IMPORTANT: From this point forward do not let the tissue sections dry out.</b></p> <hr/>

Step	Action										
<b>Step 7. Protease Digestion and Fixation</b>  <b>50 min</b>	<p><b>A.</b> Set the humidified incubator to 40 °C and verify bottom tray is filled with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working Protease Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Protease QF</td><td>4 µL</td></tr> <tr> <td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Leave slide 1 on the lab bench as it is excluded from this step.</p> <p><b>D.</b> Fill a clear staining dish with 200 mL of prewarmed 1X PBS.</p> <p><b>E.</b> Transfer the slide rack to the clear staining dish containing the prewarmed 1X PBS. Incubate for 5 min.</p> <p><b>F.</b> Working with one slide at a time, remove slide 4 and decant the 1X PBS, place slide onto the aluminum slide rack and then add 400 µL of the Working Protease Solution onto the tissue section. Repeat for slides 8 and 11.</p> <p><b>G.</b> Place the aluminum slide rack into the humidified oven and incubate for 40 min at 40 ± 1 °C.</p> <p><b>H.</b> Repeat <a href="#">Step F</a> with slides 3, 6, 7, and 10 on a separate aluminum slide rack and incubate the slides at 40 ± 1 °C in the humidified incubator for 20 min.</p> <p><b>I.</b> Repeat <a href="#">Step F</a> with slides 2, 5, and 9 on a separate aluminum slide rack and incubate the slides at 40 ± 1 °C in the humidified incubator for 10 min.</p> <p><b>J.</b> Once incubations are completed, remove the aluminum slide racks, decant the Working Protease Solution from all slides, insert them into the slide rack and transfer the slide rack into clear staining dish with 200 mL 1X PBS and gently wash by moving slide rack up and down for 1 min. Slides can be stored in 1X PBS at RT while waiting for other slides to complete.</p> <p><b>K.</b> Retrieve slide 1 and add to slide rack in 1X PBS. There should be 11 slides in the slide rack.</p> <hr/> <p><b>IMPORTANT: Process ONE slide at a time to avoid drying.</b></p> <hr/> <p><b>L.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>M.</b> Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.</p> <p><b>N.</b> Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.</p> <p><b>O.</b> Transfer the slide rack from the 4% formaldehyde solution to the clear staining dish containing 1X PBS, and incubate for 1 min. with frequent agitation.</p> <p><b>P.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>Q.</b> Transfer the 4% formaldehyde solution to a 200 mL capacity container and keep for later use.</p> <p><b>R.</b> Proceed to <a href="#">Step 8. Target Probe Set Hybridization on page 49</a> to continue the assay procedure.</p>	Working Protease Solution per Slide		Reagent	Volume	Protease QF	4 µL	1X PBS (prewarmed to 40 °C)	396 µL	Total volume	400 µL
Working Protease Solution per Slide											
Reagent	Volume										
Protease QF	4 µL										
1X PBS (prewarmed to 40 °C)	396 µL										
Total volume	400 µL										

## Typical Results

In the following example, Rat kidney FFPE tissue sections were probed with Arbp Probe Set and nuclei stained with hematoxylin following the pretreatment assay optimization procedure. Slides examined under bright field at 40X power. Rat Arbp is expressed uniformly in rat kidney FFPE tissue sections.

## Optimal Conditions

Optimal conditions were observed in slide 6 which was boiled for 10 minutes and treated with protease for 20 min. Look for a balance between signal strength, uniformity of staining and cell morphology.

**Table A.2** Typical Results of Pretreatment Assay Optimization

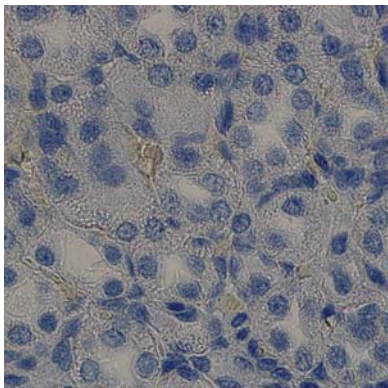

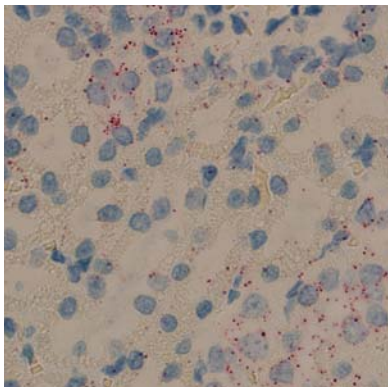
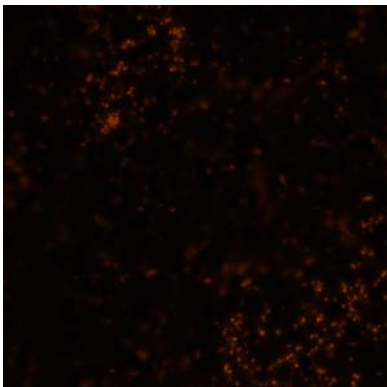
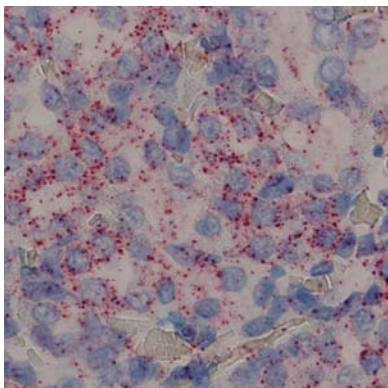
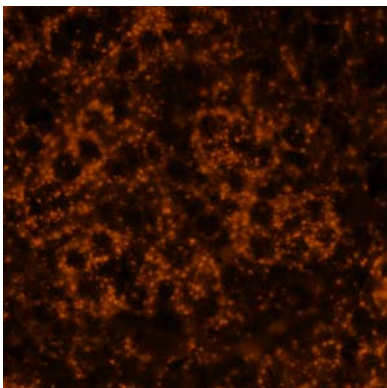
Protease Digestion Time (min)	Pretreatment Boiling Time (min)	Bright Field Image	Fluorescent Image	Results Interpretation
0	0			Untreated morphology reference slide
10	5			Cells have good morphology, signal is weak and non-ubiquitous
10	10			Cells have good morphology but signal is non-ubiquitous

Table A.2 Typical Results of Pretreatment Assay Optimization

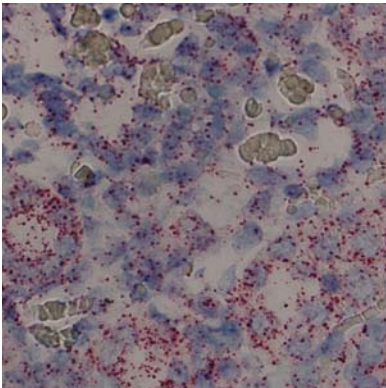
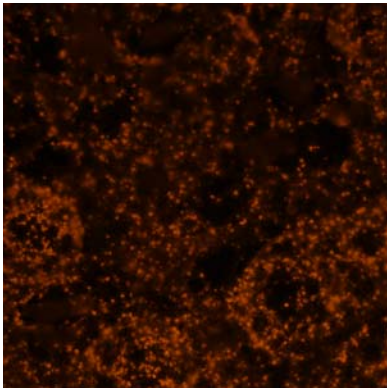
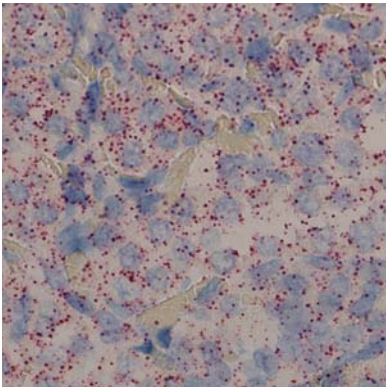
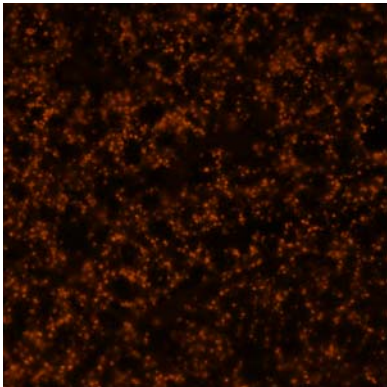
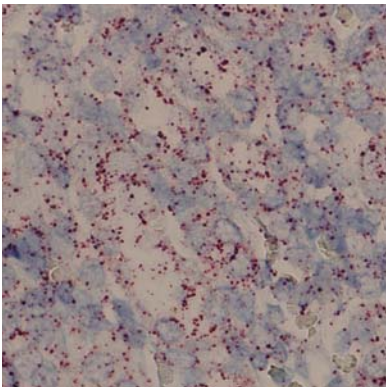
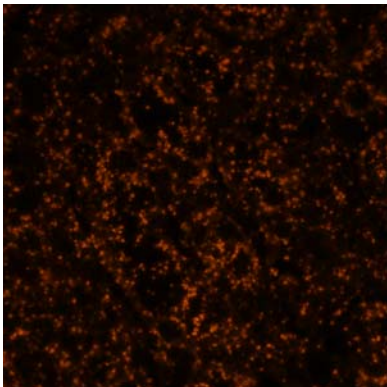
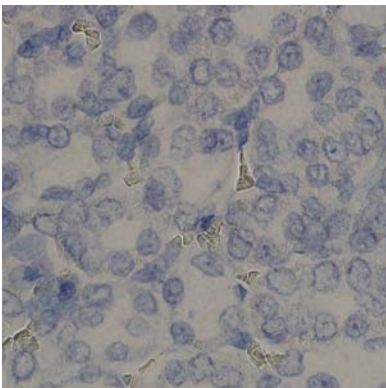
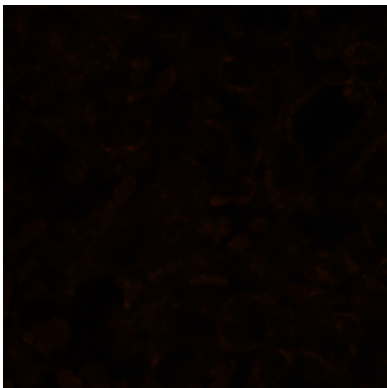
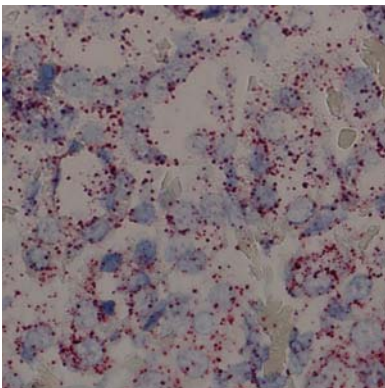
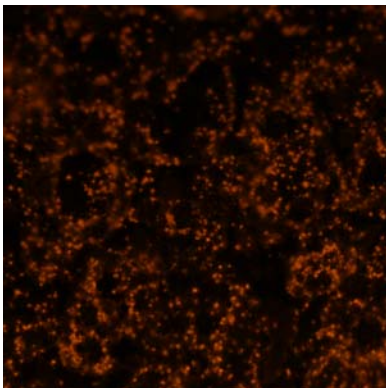
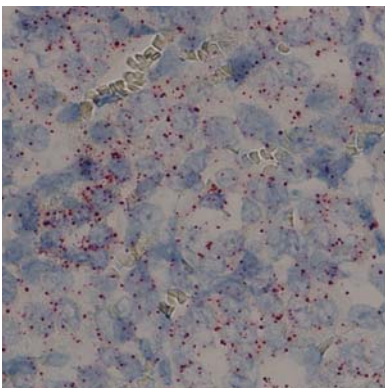
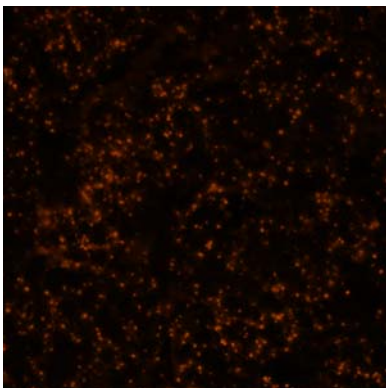
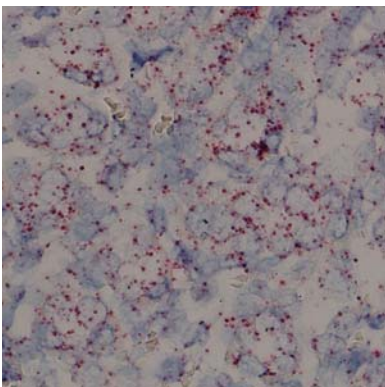
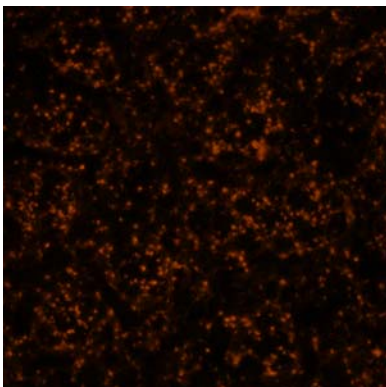
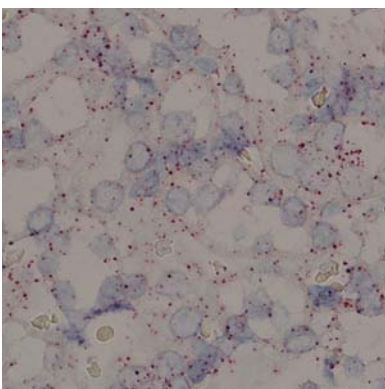
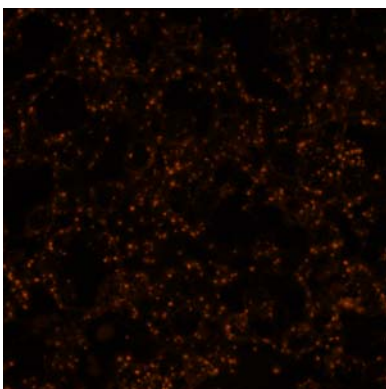
Protease Digestion Time (min)	Pretreatment Boiling Time (min)	Bright Field Image	Fluorescent Image	Results Interpretation
10	20			Cells have good morphology, signal is strong but non-ubiquitous
20	5			Cells have good morphology, signal is strong but non-ubiquitous
20	10			Cells have good morphology, signal is strong and ubiquitous
20	10			Background reference slide



Table A.2 Typical Results of Pretreatment Assay Optimization

Protease Digestion Time (min)	Pretreatment Boiling Time (min)	Bright Field Image	Fluorescent Image	Results Interpretation
20	20			Cells have good morphology, signal is strong but non-ubiquitous
40	5			Cells have poor morphology (over-digested), signal is weak and non-ubiquitous
40	10			Cells have poor morphology (over-digested), signal is weak and non-ubiquitous
40	20			Cells have poor morphology (over-digested) and signal is weak



## Pretreatment Assay Optimization Lookup Table

The table below contains a list of the tissues that we prepared according to the guidelines outlined in this manual ([Tissue Preparation Guidelines on page 7](#)) and optimized using the recommended pretreatment assay optimization procedure. You can use this table as a guideline to minimize the number of conditions if you do not have sufficient slides to perform the recommended pretreatment optimization procedure. If more than one condition is optimal, you can run both conditions and choose the one that shows the best cell morphology and signal strength. You must also include a negative control slide (without Probe Set) to ensure no background is visible and well-defined cell morphology is achieved.

**Table B.1** Treatment Optimization Table

Tissue Information		Optimal Conditions	
Species	Type (Vendor)	Boiling at 95-100 °C (min)	Protease at 40 ± 1 °C (min)
Human	Brain	20	40
	Breast	30	20
	Colon (ILS)	5	20
	Colon (Pantomics)	5	20
	Colon (USA Biomax)	5	20
	Liver	20	20
	Lung (ILS)	10	20
	Lung (Pantomics)	10	20
	Lung (USA Biomax)	10	20
	Osteoarthritic tissue	20	20
	Pancreas	10	10
	Prostate	10	20
	Prostate (Pantomics)	20	10
	Prostate (USA Biomax)	20	10
	Salivary gland	10	10
	Skin	5	10
	Tonsil	10	20
	Thyroid	10	20
Rat	Kidney	10	20
	Kidney	20	20
	Liver	10	20
	Thyroid	10	20

**Table B.1** Treatment Optimization Table

Tissue Information		Optimal Conditions	
Species	Type (Vendor)	Boiling at 95-100 °C (min)	Protease at 40 ± 1 °C (min)
Mouse	Bone	20	20
	Brain	10	10
	Heart	10	40
	Kidney	20	20
	Liver	20	20
	Lung	10	20
	Retina	10	10
Salmon	Heart	10	10
	Muscle	10	20



# QuantiGene ViewRNA ISH Tissue Assay Procedure using a Dry Oven and Humidified Incubator

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## About the QuantiGene ViewRNA ISH Tissue Assay Procedure

The QuantiGene ViewRNA ISH Tissue Assay can be run in a single long day or broken up over 2 days for added flexibility. There is an optional assay stop point, following the target probe hybridization, where the assay can be stopped and work can be continued the following day.

For convenience, the procedure is sectioned into two parts:

- Part 1: Sample Preparation and Target Probe Set Hybridization
- Part 2: Signal Amplification and Detection

with the stop point at the end of Part 1. We do not recommend stopping at any other point in the assay.


## Important Procedural Notes and Guidelines

- Perform all steps in a well-ventilated area at room temperature unless otherwise noted.
- Procedure assumes running a maximum of 12 slides at a time.
- Maximum tissue area for this procedure is 30 mm x 22 mm.
- The QuantiGene ViewRNA ISH Tissue Assay is optimized for chromogenic visualization under bright field microscope. Detection using a fluorescent microscope may require additional optimization.
- Do not mix and match kit components from different lots.
- Before beginning procedure, know the pretreatment boiling time and protease digestion time (optimized conditions) for your sample type. If you do not know these optimized conditions, refer to [Recommended Pretreatment Assay Optimization on page 7](#).
- Use a pencil to label all slides.
- Always use freshly prepared solutions in the Tissue Tek Staining and Clearing Agent dishes. Do not save or reuse solutions unless directed to do so (for example with the 4% formaldehyde solution).
- The Tissue Tek Staining Dish (clear) will be referred to as the “clear staining dish” throughout the assay procedure.
- The Tissue Tek Clearing Agent Dish (green) will be referred to as the “green clearing agent dish” throughout the assay procedure.
- Throughout the procedure, dedicate one clear staining dish for fixing in formaldehyde (we recommend labeling this dish). The other two clear staining dishes can be used interchangeably for: 1X PBS, 95% ethanol, Wash Buffer and Storage Buffer. Rinse staining dishes in between steps with ddH<sub>2</sub>O.
- The tissue culture incubator without CO<sub>2</sub> will be referred to as a humidified incubator throughout this assay procedure.
- Validate the temperature of the dry oven using the QuantiGene View Temperature Validation Kit (Affymetrix P/N QV0523).
- Validate the temperature and humidity (should be  $\geq 65\%$ ) of the humidified incubator with a temperature/humidity meter (Fisher Scientific P/N 11-661-19).
- Unless otherwise stated, all reagents prepared should be stored at room temperature until use.
- Typical processing times included in the assay procedure assume that preparation for the following step is being done during the incubation periods.
- Before opening reagents supplied in the microfuge tubes, briefly centrifuge to collect contents at the bottom of the tube.
- Discard all reagents in accordance with local, state, and federal laws.

## Part 1: Sample Preparation and Target Probe Hybridization

### Part 1 Procedure

Step	Action
<b>Step 1. Bake Slides</b>  35 min	<p><b>A.</b> Use a pencil to label the slides.</p> <p><b>B.</b> Set dry oven at <math>60 \pm 1^\circ\text{C}</math>, insert slides into slide rack, and bake the slides for 30 min.</p> <hr/> <p><b>NOTE:</b> This increases tissue attachment to the slide.</p> <hr/>
<b>Step 2. Prepare Buffers and Reagents While Slides Bake</b>  0 min	<p><b>A.</b> Prepare 2 L of 1X PBS: To a 2 L container add 200 mL of 10X PBS and 1.8 L ddH<sub>2</sub>O.</p> <p><b>B.</b> Prepare 10% formaldehyde in 1X PBS under a fume hood:</p> <hr/> <p><b>WARNING:</b> Formaldehyde is a poison and irritant. Avoid contact with skin and mucous membranes.</p> <hr/> <p>To a 200 mL capacity container add 146 ml 1X PBS and 54 mL of 37% formaldehyde and mix well.</p> <p><b>C.</b> Prepare 4% formaldehyde in 1X PBS: To a 200 mL capacity container add 22 mL of 37% formaldehyde to 178 mL 1X PBS and mix well.</p> <p><b>D.</b> Prepare 3 L of Wash Buffer: To a 3 L capacity container add components in the following order to prevent precipitation from forming and then mix well: 2.5 L ddH<sub>2</sub>O, 27 mL Wash Comp 1, 7.5 mL Wash Comp 2, and ddH<sub>2</sub>O to 3 L.</p> <p><b>E.</b> Ensure availability of:</p> <ul style="list-style-type: none"> <li>■ 400 mL 95% ethanol</li> <li>■ 400 mL ddH<sub>2</sub>O</li> <li>■ 200 mL HistoClear</li> </ul> <p><b>F.</b> Pour solutions into Tissue Tek dishes:</p> <ul style="list-style-type: none"> <li>■ In a fume hood, pour 200 mL of 10% formaldehyde into clear staining dish</li> <li>■ In a fume hood, pour 200 mL of HistoClear into green Clearing Agent Dish</li> <li>■ Pour 200 mL of 95% ethanol into clear staining dish</li> <li>■ Pour 200 mL of 1X PBS into clear staining dish</li> </ul> <p><b>G.</b> Prewarm 40 mL of 1X PBS and Probe Set Diluent QF to <math>40 \pm 1^\circ\text{C}</math>.</p> <p><b>H.</b> Thaw Probe Set(s). Place on ice until use.</p> <p><b>I.</b> Prepare 500 mL of 1X Pretreatment Solution in a 1 L glass beaker: Dilute 5 mL of 100X Pretreatment Solution in 495 mL ddH<sub>2</sub>O.</p> <p><b>J.</b> Prepare 500 mL of Storage Buffer (for Optional Stop Point): To a 200 mL container add 60 mL of Wash Comp 2 to 140 mL ddH<sub>2</sub>O and mix well.</p>
<b>Step 3. Fix Slides</b>  1 hr 5 min	<p><b>A.</b> Insert slides into an empty slide rack and submerge into a clear staining dish containing 10% formaldehyde. Incubate for 1 hour at room temperature (RT) under a fume hood.</p> <p><b>B.</b> Remove the slide rack from the 10% formaldehyde and submerge it into a clear staining dish containing 200 mL of 1X PBS. Incubate for 1 min. with frequent agitation.</p> <p><b>C.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and incubate for 1 min. with frequent agitation.</p> <p><b>D.</b> Remove each slide and decant the 1X PBS by flicking and placing it on its edge on a laboratory wipe. Place the slides on a paper towel to air dry. Make sure the slides are completely dry before going to the next step.</p> <p><b>E.</b> Set dry oven to <math>80^\circ\text{C}</math>.</p>

Step	Action
<b>Step 4. Deparaffinization</b>  <b>30 min</b>	<p><b>A.</b> Insert an empty slide rack into a green clearing agent dish containing 200 mL HistoClear solution. Cover the dish with the lid and bring it next to the dry oven.</p> <p><b>B.</b> Bake the slides in the dry oven at 80 °C for 3 min. The paraffin should melt as soon as the slides are in the dry oven.</p> <p><b>C.</b> Remove lid from green Tissue Tek dish, and immediately insert the warm slides in the HistoClear solution, cover the dish with the lid and incubate under a fume hood at RT for 10 min. with frequent agitation.</p> <p><b>D.</b> Lift the slide rack from the HistoClear solution and submerge into the clear staining dish containing 200 mL 95% ethanol and incubate for 1 min. with frequent agitation.</p> <p><b>E.</b> Decant the 95% ethanol, refill with 200 mL of fresh 95% ethanol, and incubate for 1 min. with frequent agitation.</p> <p><b>F.</b> Remove the slides from the slide rack and place them on a paper towel to air dry for 5 min at RT. Discard the 95% ethanol.</p>
<b>Step 5. Draw Hydrophobic Barrier</b>  <b>1 hr</b>	<p><b>A.</b> Dab the hydrophobic pen on a paper towel several times before use to ensure proper flow of the hydrophobic solution.</p> <p><b>B.</b> To create a hydrophobic barrier, place the slide over the template image below, tissue sections should fall inside blue rectangle, and lightly trace the blue rectangle 2 to 4 times with the ImmEdge Hydrophobic Barrier Pen. It may be necessary to draw the hydrophobic barrier over tissue edges for larger sections or sections mounted close to the edge of the slide. Allow for barrier to dry at RT for 20-30 min.</p> <hr/> <p><b>IMPORTANT: Consistently draw hydrophobic barrier size as indicated in template, even if using smaller tissue sections.</b></p> <hr/> <p><b>IMPORTANT: Draw the barrier 2-4 times to ensure a solid seal.</b></p> <hr/> 
<b>Step 6. Tissue Pretreatment</b>  <b>10-25 min, depending on optimized time</b>	<p><b>A.</b> Bring 500 mL of 1X Pretreatment Solution to boil (100 °C) in a 1 L beaker tightly covered with aluminum foil on a hot plate. When boiling is reached, use a water-proof probe thermometer to measure and maintain the boiling temperature at 95-100 °C.</p> <p><b>B.</b> Load the slides into the slide rack.</p> <p><b>C.</b> Using a pair of forceps, submerge the slide rack into the boiling 1X Pretreatment Solution. Cover the glass beaker with aluminum foil and incubate at 95-100 °C for the optimal time as determined in <a href="#">Pretreatment Assay Optimization Procedures and Typical Results on page 31</a>.</p> <p><b>D.</b> Using a pair of forceps, remove the slide rack loaded with slides and submerge it into a clear staining dish containing 200 mL ddH<sub>2</sub>O. Incubate for 1 min with frequent agitation.</p> <p><b>E.</b> Decant the ddH<sub>2</sub>O and refill the clear staining dish with fresh ddH<sub>2</sub>O. Incubate for 1 min. with frequent agitation.</p> <hr/> <p><b>IMPORTANT: From this point forward do not let the tissue sections dry out.</b></p> <hr/>

Step	Action										
<b>Step 7. Protease Digestion and fixation</b>  <b>30-50 min, depending on optimized time</b>	<p><b>A.</b> Set the humidified incubator to 40 °C and verify the bottom tray is filled with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Protease Solution by diluting the Protease QF 1:100 in prewarmed 1X PBS. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working Protease Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Protease QF</td><td>4 µL</td></tr> <tr> <td>1X PBS (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Remove slides from slide rack, flick off excess ddH<sub>2</sub>O and place them onto an aluminum slide rack.</p> <p><b>D.</b> Add 500 µL of prewarmed 1X PBS to each slide and incubate in the humidified incubator for 3 min. at 40 ± 1 °C.</p> <p><b>E.</b> Working with one slide at a time, decant the 1X PBS, place the slide onto the aluminum slide rack, and then add 400 µL of the Working Protease Solution onto the tissue section. Repeat for the rest of the slides.</p> <hr/> <p><b>IMPORTANT: Make sure every slide is incubated for the full duration of time determined by the Pretreatment Assay Optimization Procedure. Process ONE slide at a time to avoid drying.</b></p> <hr/> <p><b>F.</b> Place the aluminum slide rack into the humidified incubator and incubate at 40 ± 1 °C for the optimal time as determined in the <a href="#">Pretreatment Assay Optimization Procedures and Typical Results on page 31</a>.</p> <p><b>G.</b> After incubation, decant the Working Protease Solution from the slides, insert them into the slide rack and transfer slide rack into clear staining dish with 200 mL 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>H.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>I.</b> Transfer the slide rack into the clear staining dish containing 4% formaldehyde and incubate under a fume hood for 5 min at RT.</p> <p><b>J.</b> Decant the clear staining dish containing 1X PBS and refill with 200 mL of fresh 1X PBS.</p> <p><b>K.</b> Transfer the slide rack from the 4% formaldehyde solution to the clear staining dish containing 1X PBS, and incubate for 1 min. with frequent agitation.</p> <p><b>L.</b> Decant the 1X PBS, refill with 200 mL of fresh 1X PBS and gently wash by moving slide rack up and down for 1 min.</p> <p><b>M.</b> Transfer the 4% formaldehyde solution to a 200 mL capacity container, keep for later use.</p>	Working Protease Solution per Slide		Reagent	Volume	Protease QF	4 µL	1X PBS (prewarmed to 40 °C)	396 µL	Total volume	400 µL
Working Protease Solution per Slide											
Reagent	Volume										
Protease QF	4 µL										
1X PBS (prewarmed to 40 °C)	396 µL										
Total volume	400 µL										

Step	Action																				
<b>Step 8. Target Probe Set Hybridization</b>  <b>3 hr 10 min</b>	<p><b>A.</b> Set the humidified incubator to 40 °C and verify the bottom tray is filled with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working Probe Set Solutions by diluting the QuantiGene ViewRNA Probe Set(s) 1:50 in prewarmed Probe Set Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <hr/> <p><b>IMPORTANT: We recommend running assay controls, 1 positive and 1 negative control slide, to ensure proper signal is obtained.</b></p> <hr/> <table><tr><th>Working Probe Set Solution per Slide</th><th>Target Sample</th><th>Negative Control</th><th>Positive Control</th></tr><tr><th>Reagent</th><th colspan="3">Volume</th></tr><tr><td>Probe Set Diluent (prewarmed to 40 °C)</td><td>392 µL</td><td>400 µL</td><td>392 µL</td></tr><tr><td>QuantiGene ViewRNA TYPE 1 Probe Set</td><td>8 µL*</td><td>0†</td><td>8 µL‡</td></tr><tr><td>Total volume</td><td>400 µL</td><td>400 µL</td><td>400 µL</td></tr></table> <p>* Use target Probe Set</p> <p>† Do not include a target Probe Set</p> <p>‡ Use positive control Probe Set</p> <p><b>C.</b> Remove each slide from 1X PBS and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe.</p> <p><b>D.</b> Place the slides on the aluminum slide rack and immediately add 400 µL Working Probe Set Solution to each tissue section.</p> <p><b>E.</b> Place the slides on the aluminum slide rack, then into the humidified incubator, and incubate at 40 ± 1 °C for 3 hr.</p>	Working Probe Set Solution per Slide	Target Sample	Negative Control	Positive Control	Reagent	Volume			Probe Set Diluent (prewarmed to 40 °C)	392 µL	400 µL	392 µL	QuantiGene ViewRNA TYPE 1 Probe Set	8 µL*	0†	8 µL‡	Total volume	400 µL	400 µL	400 µL
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QuantiGene ViewRNA TYPE 1 Probe Set	8 µL*	0†	8 µL‡																		
Total volume	400 µL	400 µL	400 µL																		
<b>Step 9. Wash Slides</b>  <b>10 min</b>	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Probe Set Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>																				
<b>Step 10. Optional Stopping Point</b>  <b>1 min</b>	<p><b>A.</b> Store slides in a clear staining dish containing 200 mL of Storage Buffer for up to 24 hours at RT.</p> <p><b>B.</b> The following reagent preparations should be stored at RT for use in Part 2:</p> <ul style="list-style-type: none"><li>■ 4% formaldehyde</li><li>■ 1X PBS</li><li>■ Wash Buffer</li></ul> <p><b>C.</b> All other reagent and solution preparations should be discarded.</p> <p><b>D.</b> When you are ready to continue with the assay, proceed to <a href="#">Step 12. Prepare Additional Buffers and Reagents on page 50.</a></p>																				

## Part 2: Signal Amplification and Detection

### Part 2 Procedure

Step	Action										
<b>Step 11. Preparation for Part 2</b>  5 min	<p>If you paused the assay after Part 1, complete this step, otherwise go to <a href="#">Step 12. Prepare Additional Buffers and Reagents on page 50</a>.</p> <p><b>A.</b> Remove the slides from Storage Buffer, transfer slide rack to clear staining dish containing Wash Buffer, and incubate for 1 min with frequent agitation.</p> <p><b>B.</b> Decant Wash Buffer, refill with 200 mL fresh Wash Buffer, and incubate for 1 min. with frequent agitation.</p>										
<b>Step 12. Prepare Additional Buffers and Reagents</b>  10 min	<p><b>A.</b> Prepare 1 L of 0.01% ammonium hydroxide in ddH<sub>2</sub>O: In a fume hood, add 0.33 mL 30% ammonium hydroxide to 999.67 mL ddH<sub>2</sub>O and mix well.</p> <p><b>B.</b> Ensure availability of 200 mL Gill's Hematoxylin.</p> <p><b>C.</b> If you plan on using fluorescent detection, prepare 200 mL DAPI. The final dilution of DAPI should be 0.5 µg/mL in 1X PBS.</p> <p><b>D.</b> Prewarm Amplifier Diluent QF and Label Probe Diluent QF buffers to 40 °C.</p> <p><b>E.</b> Thaw PreAmp1 QF and Amp1 QF. Place on ice until use.</p> <p><b>F.</b> Place Label Probe-AP on ice.</p> <p><b>G.</b> Bring Fast Red Tablets, Naphthol Buffer and AP Enhancer Solution to RT.</p>										
<b>Step 13. PreAmplifier Hybridization</b>  35 min	<p><b>A.</b> Set the humidified incubator to 40 °C and verify the bottom tray is filled with ddH<sub>2</sub>O.</p> <p><b>B.</b> Using the table below as a guide, prepare the Working PreAmp1 Solution by diluting PreAmp1 QF 1:100 in prewarmed Amplifier Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table border="1"> <thead> <tr> <th colspan="2">Working PreAmp1 Solution per Slide</th></tr> <tr> <th>Reagent</th><th>Volume</th></tr> </thead> <tbody> <tr> <td>Amplifier Diluent QF (prewarmed to 40 °C)</td><td>396 µL</td></tr> <tr> <td>PreAmp1 QF</td><td>4 µL</td></tr> <tr> <td>Total volume</td><td>400 µL</td></tr> </tbody> </table> <p><b>C.</b> Remove each slide and decant the Wash Buffer by flicking and placing the slide on its edge on a laboratory wipe. Place slides on aluminum slide rack and immediately add 400 µL of Working PreAmp1 Solution to each tissue section.</p> <p><b>D.</b> Place the slides on the aluminum slide rack, then into the humidified incubator, and incubate at 40 ± 1 °C for 25 min.</p> <hr/> <p><b>IMPORTANT: Incubation time should not exceed 25 min.</b></p> <hr/>	Working PreAmp1 Solution per Slide		Reagent	Volume	Amplifier Diluent QF (prewarmed to 40 °C)	396 µL	PreAmp1 QF	4 µL	Total volume	400 µL
Working PreAmp1 Solution per Slide											
Reagent	Volume										
Amplifier Diluent QF (prewarmed to 40 °C)	396 µL										
PreAmp1 QF	4 µL										
Total volume	400 µL										
<b>Step 14. Wash Slides</b>  10 min	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working PreAmp1 Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>										

Step	Action																				
<b>Step 15. Amplifier Hybridization</b>  20 min	<p><b>A.</b> Using the table below as a guide, prepare the Working Amp1 Solution by diluting Amp1 QF, 1:100 in prewarmed Amplifier Diluent QF and briefly vortex. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table><tr><th colspan="2">Working Amp1 Solution per Slide</th></tr><tr><th>Reagent</th><th>Volume</th></tr><tr><td>Amplifier Diluent QF (prewarmed to 40 °C)</td><td>396 µL</td></tr><tr><td>Amp1 QF</td><td>4 µL</td></tr><tr><td>Total volume</td><td>400 µL</td></tr></table> <p><b>B.</b> Remove each slide from Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides on an aluminum slide rack and immediately add 400 µL of Working Amp1 Solution to each tissue section.</p> <p><b>C.</b> Place the slides on the aluminum slide rack, then into the humidified incubator, and incubate at 40 ± 1 °C for 15 min.</p> <hr/> <p><b>IMPORTANT: Incubation time should not exceed 15 min.</b></p> <hr/>	Working Amp1 Solution per Slide		Reagent	Volume	Amplifier Diluent QF (prewarmed to 40 °C)	396 µL	Amp1 QF	4 µL	Total volume	400 µL										
Working Amp1 Solution per Slide																					
Reagent	Volume																				
Amplifier Diluent QF (prewarmed to 40 °C)	396 µL																				
Amp1 QF	4 µL																				
Total volume	400 µL																				
<b>Step 16. Wash Slides</b>  10 min	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Amp1 Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 2 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 2 min. with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>																				
<b>Step 17. Label Probe-AP Hybridization</b>  25 min	<p><b>A.</b> Using the table below (left side) as a guide, prepare 1:10 Working Label Probe-AP Solution by diluting Label Probe-AP 1:10 in prewarmed Label Probe Diluent QF and briefly vortexing to mix.</p> <p><b>B.</b> Using the table below (right side) as a guide, prepare Working Label Probe-AP Solution by diluting the 1:10 Working Label Probe-AP Solution to 1:1000 in prewarmed Label Probe Diluent QF and briefly vortexing to mix. Scale reagents according to the number of assays to be run. Include one slide volume overage.</p> <table><tr><th colspan="2">1:10 Working Label Probe-AP Solution per Slide</th><th colspan="2">1:1000 Working Label Probe-AP Solution per Slide</th></tr><tr><th>Reagent</th><th>Volume</th><th>Reagent</th><th>Volume</th></tr><tr><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>54 µL</td><td>Label Probe Diluent QF (prewarmed to 40 °C)</td><td>396 µL</td></tr><tr><td>Label Probe-AP</td><td>6 µL</td><td>1:10 Working Label Probe-AP (from 1:10 dilution)</td><td>4 µL</td></tr><tr><td>Total volume</td><td>60 µL</td><td></td><td>400 µL</td></tr></table> <p><b>C.</b> Discard any remainder of 1:10 Working Label Probe-AP solution.</p> <p><b>D.</b> Remove each slide from the Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides on an aluminum slide rack and immediately add 400 µL of 1:1000 Working Label Probe-AP Solution to each tissue section.</p> <p><b>E.</b> Place the slides on the aluminum slide rack, into the humidified incubator and incubate at 40 ± 1 °C for 15 min.</p> <hr/> <p><b>IMPORTANT: Incubation time should not exceed 15 min.</b></p> <hr/>	1:10 Working Label Probe-AP Solution per Slide		1:1000 Working Label Probe-AP Solution per Slide		Reagent	Volume	Reagent	Volume	Label Probe Diluent QF (prewarmed to 40 °C)	54 µL	Label Probe Diluent QF (prewarmed to 40 °C)	396 µL	Label Probe-AP	6 µL	1:10 Working Label Probe-AP (from 1:10 dilution)	4 µL	Total volume	60 µL		400 µL
1:10 Working Label Probe-AP Solution per Slide		1:1000 Working Label Probe-AP Solution per Slide																			
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Label Probe-AP	6 µL	1:10 Working Label Probe-AP (from 1:10 dilution)	4 µL																		
Total volume	60 µL		400 µL																		

Step	Action
<b>Step 18. Wash Slides</b>  <b>13 min</b>	<p><b>A.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of Wash Buffer.</p> <p><b>B.</b> After incubation, decant the Working Label Probe-AP Solution from the slides and insert them into the slide rack.</p> <p><b>C.</b> Incubate the slides in Wash Buffer at RT for 3 min. with frequent agitation.</p> <p><b>D.</b> Decant the Wash Buffer, refill with 200 mL fresh Wash Buffer and incubate the slides at RT for 3 min with frequent agitation. Repeat this step one more time for a total of 3 washes.</p>
<b>Step 19. Apply Fast Red Substrate</b>  <b>1 hr</b>	<p><b>A.</b> Remove each slide from the Wash Buffer and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench.</p> <p><b>B.</b> Immediately add 400 <math>\mu</math>L of the AP-Enhancer Solution to each tissue section (pipet directly from bottle) and incubate at RT for 5 min. while preparing the Fast Red Substrate.</p> <p><b>C.</b> Prepare the Fast Red Substrate: in a 15 ml conical tube, add 5 ml of Naphthol Buffer and one Fast Red Tablet. Vortex at high speed to completely dissolve the tablet.</p> <p><b>D.</b> Decant the AP Enhancer Solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Immediately add 400 <math>\mu</math>L of Fast Red Substrate onto each tissue section.</p> <p><b>E.</b> Place the slides on the aluminum slide rack, then into the humidified incubator, and incubate at <math>40 \pm 1</math> °C for 30 min.</p> <p><b>F.</b> Insert an empty slide rack into a clear staining dish containing 200 mL of 1X PBS.</p> <p><b>G.</b> After incubation, decant the Fast Red Substrate from the slides and insert them into the slide rack.</p> <p><b>H.</b> Move the slide rack up and down several times for 1 min to rinse off the Fast Red Substrate.</p> <p><b>I.</b> Retrieve 200 mL of 4% formaldehyde (used previously) and place in the clear staining dish labeled for formaldehyde.</p> <p><b>J.</b> Move the slide rack to the clear staining dish containing 200 mL of 4% formaldehyde and incubate for 5 min under a fume hood.</p> <p><b>K.</b> Rinse off the residual formaldehyde by transferring the slide rack to a clear staining dish containing fresh 1X PBS. Move the slide rack up and down several times for 1 min.</p>
<b>Step 20. Counterstain</b>  <b>50 min</b>	<p><b>A.</b> Remove each slide from 1X PBS and decant the solution by flicking and briefly placing the slide on its edge on a laboratory wipe. Place slides flat on the lab bench and add 500 <math>\mu</math>L of Gill's Hematoxylin Solution. Incubate the slides for 5 min at RT.</p> <p><b>B.</b> Insert the empty slide rack into a clear staining dish containing ddH<sub>2</sub>O.</p> <p><b>C.</b> After incubation, decant Gill's Hematoxylin Solution from the slides and insert them into the slide rack containing ddH<sub>2</sub>O. Move the slide rack up and down several times to rinse off Gill's Hematoxylin Solution.</p> <p><b>D.</b> Decant the ddH<sub>2</sub>O, refill with 200 mL fresh ddH<sub>2</sub>O and move slide rack up and down several times. Repeat this step one more time.</p> <p><b>E.</b> Decant the ddH<sub>2</sub>O, refill with 200 mL 0.01% ammonium hydroxide and incubate for 10 sec.</p> <p><b>F.</b> Decant 0.01% ammonium hydroxide, refill with fresh ddH<sub>2</sub>O and move slide rack up and down several times.</p> <p><b>G.</b> <i>Optional.</i> If you plan to view slides using the fluorescent microscope, then move slide rack into a clear staining dish containing 200 mL DAPI staining solution. Incubate the slides for 1 min. Decant DAPI staining solution, refill with 200 mL fresh ddH<sub>2</sub>O and move the slide rack up and down several times to rinse off DAPI solution. DAPI staining step must be performed after the Gill's Hematoxylin staining step.</p> <p><b>H.</b> Remove the slides from the slide rack and decant the ddH<sub>2</sub>O by flicking and briefly placing the slide on its edge on a laboratory wipe. Place them onto a paper towel to air dry.</p> <p><b>I.</b> Ensure that slide sections are completely dry before mounting (about 20 min).</p>



Step	Action
<p><b>Step 21. Add Coverslip and Image</b></p> <p>20 min</p>	<p><b>A.</b> Add a minimum of two drops of DAKO Ultra Mount mounting medium to tissue section without making any bubbles. Cover the slide section with a piece of 24 mm x 55 mm cover glass.</p> <p><b>B.</b> Dab off the excess mounting medium and image the result under a bright field microscope and/or fluorescent microscope.</p> <hr/> <p><b>IMPORTANT:</b> Slides should be viewed and imaged within one week of mounting to ensure the accuracy and validity of the results. Bubbles may start to form after one week making imaging difficult. Please refer to <a href="#">Remounting Slides After Bubbles Form on page 55</a> for the remounting procedure.</p> <hr/> <p><b>NOTE:</b> Fluorescence signals will fade over time. However, chromogenic signal will remain stable for at least 3 months.</p> <hr/> <p><b>C.</b> 0.01% ammonium hydroxide solution can be stored at RT for up to one month. Discard all remaining reagent and solution preparations.</p>



## Remounting Slides After Bubbles Form

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During storage, bubbles may start to form on mounted slides. This appendix provides a procedure to remove the bubbles and remount the slides.

Step	Action
Remount Slides	<p><b>A.</b> Fill a clear staining dish with 200 mL 1X PBS.</p> <p><b>B.</b> Insert slides into a slide rack and submerge into 1X PBS.</p> <p><b>C.</b> Incubate slides at RT for 24-48 hr to allow for coverslips to fall off.</p> <p><b>D.</b> Agitate the slide rack for 1 min, coverslips should fall off. If coverslips do not fall off, increase incubation time.</p> <p><b>E.</b> Transfer the slide rack to a clear staining dish filled with 200 mL ddH<sub>2</sub>O and move the rack up and down several times to rinse.</p> <p><b>F.</b> Decant ddH<sub>2</sub>O, refill with 200 mL ddH<sub>2</sub>O and move slide rack up and down several times to rinse.</p> <p><b>G.</b> Remove slides from slide rack and set on the lab bench to air dry.</p> <p><b>H.</b> Mount slides as follows</p> <ol style="list-style-type: none"><li>1) Add a minimum of two drops of DAKO Ultra Mount mounting medium to tissue section without making any bubbles. Cover the slide section with a piece of 24 mm x 55 mm cover glass.</li><li>2) Dab off any excess mounting medium and observe the result under a bright field microscope.</li></ol> <p><b>IMPORTANT:</b> Slides should be viewed and imaged within one week of mounting to ensure the accuracy and validity of the results. Bubbles may start to form after one week making imaging difficult.</p>



## Validated Imaging Systems for QuantiGene ViewRNA ISH Tissue Assay

The QuantiGene ViewRNA ISH Tissue Assay uses the proprietary Fast Red substrate. Conversion of the Fast Red substrate by alkaline phosphatase generates a red precipitate that has both chromogenic (red) and fluorescent (ex/em = 550/570) properties. The red precipitate appears as distinct dots clearly visible at magnifications of 20X or more. We have found the following microscope setups and imaging systems to produce optimal bright field or fluorescent tissue images. Other imaging systems might produce similar results. We recommend that you ask the vendor for a comparable setup and demonstration.

Descriptions + Applications		Microscope	Lens and Filters	Camera
<b>Standard Microscope</b>				
Bright field Microscope	Standard bright field microscope Used for quick viewing	Leica DM 2000	20x/NA 0.50 HCX FLUOTAR and 40x/NA 0.75 FLOUTAR or similar lens	NA
Digital Camera Microscope Combo CISH/FISH	Used best for viewing + capturing color image Okay quality for fluorescent mode  Software is relatively easy to use	Olympus IX71 or similar system	20x/NA 0.75 40x/NA 1.30 or similar lens  Filters Fast Red/ Ex/Em= 550/570 DAPI/Ex/Em = 360/460	Olympus DP71 Best for CISH OK for FISH
<b>Digital Pathology Automated Slide Scanner</b>				
Brightfield/CISH Scanner	Automated slide scanner + Digital Pathology + data management system SW	Aperio ScanScope XT	Lens 20x or 40x objective	NA
CISH+FISH Scanner	Same as above	Metafer/MetaSystem	Lens 20x or 40x objective  Filters Fast Red/ Ex/Em= 550/570 DAPI/Ex/Em = 360/460	NA
CISH + FISH Scanner	Same as above	Nanozoomer/Olympus/ Hamamatsu	Line scanner  Filters Fast Red/ Ex/Em= 550/570 DAPI/Ex/Em = 360/460	TDI Sensor



## Templates for Drawing the Hydrophobic Barrier



**NOTE:** To ensure templates print to the correct size, make sure that you select none under the page scaling option in the print dialog box.

**Figure F.1** Tissue Slide Templates

